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Regulation of Vancomycin Resistance and Stress Response by the *MSAABCR* Operon in *Staphylococcus aureus*

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REGULATION OF VANCOMYCIN RESISTANCE AND STRESS RESPONSE BY THE *MSAABCR* OPERON IN *STAPHYLOCOCCUS AUREUS*

by

Dhritiman Samanta

Abstract of a Dissertation
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
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ABSTRACT

REGULATION OF VANCOMYCIN RESISTANCE

AND STRESS RESPONSE BY THE *MSAABCR*

OPERON IN *STAPHYLOCOCCUS AUREUS*

by Dhritiman Samanta

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Staphylococcus aureus is the predominant cause of public health problems around the world. Vancomycin has been an important antibiotic against Methicillin Resistant *Staphylococcus aureus* (MRSA) infections. However, Vancomycin Intermediate *S. aureus* (VISA) strains have been reported. These strains are characterized by thick cell walls, reduced autolytic rate, reduced PBP4 activity, and increased amount of D-Ala-D-Ala termini in the cell wall. In this study, we show that the *msaABCR* operon regulates vancomycin resistance in two clinical VISA strains. Deletion of the *msaABCR* operon in strains Mu50 and HIP6297 resulted in a significant decrease in the minimum inhibitory concentration (MIC) for vancomycin. Transmission Electron Microscopic analysis showed a 50% decrease in the cell wall thickness in the mutants relative to wild types. The *msaABCR* mutant of Mu50 but not HIP6297 showed a 3-fold up-regulation of *pbp4*. Up-regulation of *pbp4* was confirmed by fluorescent penicillin–binding assay, which showed that the mutant has a 6-fold higher amount of PBP4 protein relative to wild type. Mu50 *msaABCR* mutant also showed a 6-fold down-regulation of stress response regulator *sigB*. On the other hand, the *msaABCR* mutant of HIP6297 showed a 4-fold down-regulation of *mprF*. Collectively, my data show that the *msaABCR* is required for maintaining the thickness of cell wall and vancomycin resistance in two clinical VISA

strains Mu50 and HIP6297; however, the mechanism and regulation by the *msaABCR* operon in the genetic level are strain dependent.

Staphylococcus aureus is also the predominant cause of bacteremia worldwide. The health hazard burden caused by this species is severely exacerbated by worldwide dissemination of clones resistant to beta-lactam antibiotics in hospitals and communities. The distribution of MRSA clones is dynamic and tends to be geographically unique. The purpose of the second part of my study is to determine the molecular characteristics and antibiotic resistance properties of the MRSA isolates causing bacteremia in a major hospital in south Mississippi, USA. This study allows us to associate specific clonal types with bacteremia isolates and detect outbreak by any particular type in south Mississippi.

The University of Southern Mississippi

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DEDICATION

I dedicate my dissertation to my parents, Dilip Kumar Samanta and Tripti Samanta, for their endless sacrifices, prayers, and continued support for my studies.

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CHAPTER I

INTRODUCTION

The Biology of *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is a Gram - positive coccus bacterium belonging to the phylum *Firmicutes,* class *Bacilli,* and order *Bacillales*. This coagulase positive coccus, measuring about 0.7 – 1.2 µm in diameter, is much studied because of its clinical importance to human. Although *S. aureus* normally resides in the nose, throat, armpit, groin, and intestinal tract of healthy individuals, when introduced into a wound or skin incision, cause infections. According to the Center for Disease Control and Prevention (CDC), *S. aureus* is the most common cause of hospital-acquired infections causing 80,461 invasive MRSA infections including 11,285 deaths in USA. Tremendous amount of resources have been used to understand the molecular basis of its virulence and evolutionary success using modern technology. An in depth understanding of its global regulatory network is necessary to fully decipher its array of virulence factors and to devise mechanism to control it.

The term “staphylococcus” was synthesized from the Greek word “staphyle,” which means bunch of grapes and the term “coccus” meaning grain or berry, because of its clustered appearance under microscope. Robert Koch, in 1878 first identified that abscesses are caused by Gram-positive cocci. Later in 1884, Rosenbach classified species of staphylococci depending on their capability of producing pigments, where more virulent species formed golden colonies whereas less pathogenic ones formed white colonies. Coagulase testing later proved to be a better classification of staphylococci than pigment formation. In clinic, a positive coagulase test confirms the identity of *S. aureus.*

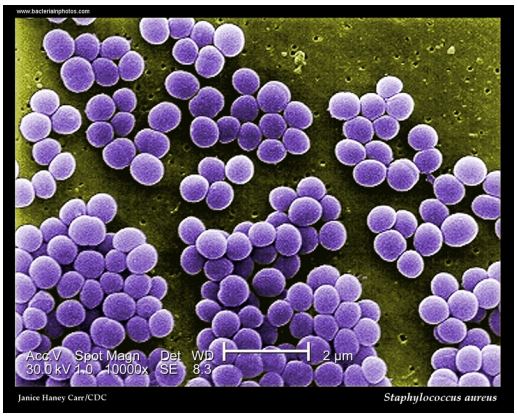

Figure 1.1. A representative image of *S. aureus* under microscope. Characteristic clumping behavior is evident in this figure. (Photo Credit: Janice Haney Carr, Center for Disease Control and Prevention)

S. aureus grows most rapidly under aerobic conditions and in the presence of carbon-di-oxide. However, *S. aureus* is also capable of surviving in environments with very low concentrations of oxygen as well, such as within a biofilm (1). It is versatile in terms of tolerating extreme conditions. For example it can tolerate high amounts of salt in growth medium (7.5 – 10% of Sodium Chloride) (2) and a wide pH range (4.8 – 9.4).

S. aureus colonizes human body in early years of life and is carried throughout the life. 30% of United States population carry *S. aureus* in the nasal passage (3, 4). It adheres to nasal mucosal surface with the help of several virulence factors such as surface protein adhesins, fibronectin – binding protein, and clumping factor (5-8). A large variety of proteins and carbohydrates expressed on the cell surface enables *S. aureus* to bind to a number of host factors such as epithelial cells, endothelial cells, platelets, and host intercellular matrix proteins (9). Penicillin, once was used to treat *S. aureus* infections. However, as *S. aureus* got resistant to it, methicillin, a penicillin derivative was introduced. *S. aureus*, however acquired resistance against methicillin quickly. Rise of Methicillin Resistant *S. aureus* (MRSA) has caused a widespread problem in public

health worldwide. MRSA causes about 1,20,000 hospitalizations and more than 1 billion dollars healthcare costs in the U.S.. Initially, MRSA strains were largely confined to hospitals and were referred to as hospital-acquired MRSA (HA-MRSA), causing treatment difficulties, especially in patients with prior exposure to antibiotics. During the past two decades, *S. aureus* infections have been worsened by the emergence of community-acquired MRSA (CA-MRSA). The United States, in particular has been facing an epidemic caused by CA-MRSA isolates. CA-MRSA isolates are clinically significant because they can also cause severe health issues in individuals who are apparently healthy and have never been exposed to any predisposing risk factors. It is imperative that new therapeutic solutions are found for this rapidly evolving pathogen.

Staphylococcus aureus is a multifaceted pathogen that causes a wide array of infections ranging from simple skin abscesses to more complex and life threatening conditions like endocarditis and osteomyelitis. Other serious conditions caused by *S. aureus* include *S. aureus* bacteremia (SAB), *S. aureus* infective endocarditis (SAIE), surgical site infections, device related infections (catheters, implants etc.), skin and soft tissue infections (SSTIs), spine and brain abscesses, ocular diseases, staphylococcal pneumonia, urinary tract infections, toxic shock syndrome, and toxin mediated syndromes. *S. aureus* is able to cause these infections by successfully utilizing an array of virulence factors. These factors are often redundant and/or variable across different strains, making it even harder to formulate effective prophylactic or immunological therapies.

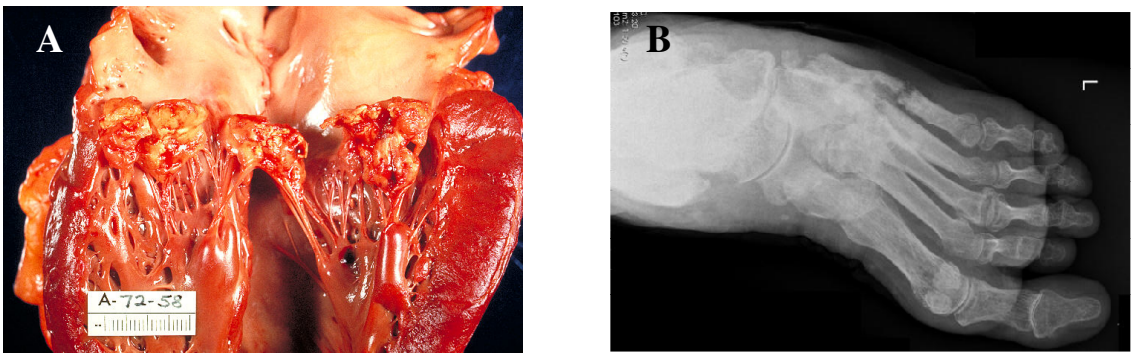

Figure 1.2. A. Endocarditis and B. Osteomyelitis. Photo credit: Center for Disease Control and Prevention)

Antibiotic Resistance in *Staphylococcus aureus*

Treatment of *S. aureus* has become more difficult attributable to the profuse increase in prevalence of MRSA strains in the last few decades. Particularly, CA-MRSA strains are of great concern because of their capability of causing wide variety of infections, high growth rate, and causing diseases in people without any risk factors. CA-MRSA strains cause skin and soft tissue infections, which spreads from skin to skin contact with infected individuals. Interestingly, CA-MRSA is resistant to only β-lactams and only a few other classes of antibiotics, however, HA-MRSA is resistant to multiple types of antibiotics (10). The antibiotic resistance profile of MRSA strains is partly due to the presence of resistance genes that are located in a mobile genetic element called Staphylococcal cassette chromosome *mec* (SCC*mec*). The methicillin resistance of *S. aureus* is the result of the presence of the *mecA* gene, which encodes a 78-kDa penicillin-binding protein (PBP2a or PBP2′). *S. aureus* possesses four types of penicillin binding proteins, which are essential enzymes for synthesis of cell wall. The β-lactam antibiotics such as penicillin and methicillin bind to PBP2 and inhibits cell wall synthesis process. PBP2a, produced by *mecA* gene in MRSA strains has a lower affinity for the β-lactam antibiotics. As a result, even in the presence of these antibiotics, the peptidoglycan

synthesis is not disrupted and the bacterium survives (11, 12). The *mecA* gene is housed within the *mec* operon along with the regulatory genes *mecI* and *mecR1* (11). Since the first detection of the SCC*mec* element from N315 in 1999, multiple *mec* types have been identified because of the hypervariability of SCC*mec* elements. Nine SCC*mec* types (I–VIII and V_T) have been detected to date. These are distinguished by the *ccr* gene complex which helps in excision and insertion of SCC*mec* elements (13-15). Clones of unique SCC*mec* types have driven the epidemic of MRSA strins worldwide. For example, SCC*mec* type I was first discovered in the United Kingdom in 1961 and spread around the world during the ’60s. Following that, in 1982, SCC*mec* type II was first identified in Japan also spread worldwide. During the 1990s SCC*mec* type IV caused a global epidemic.

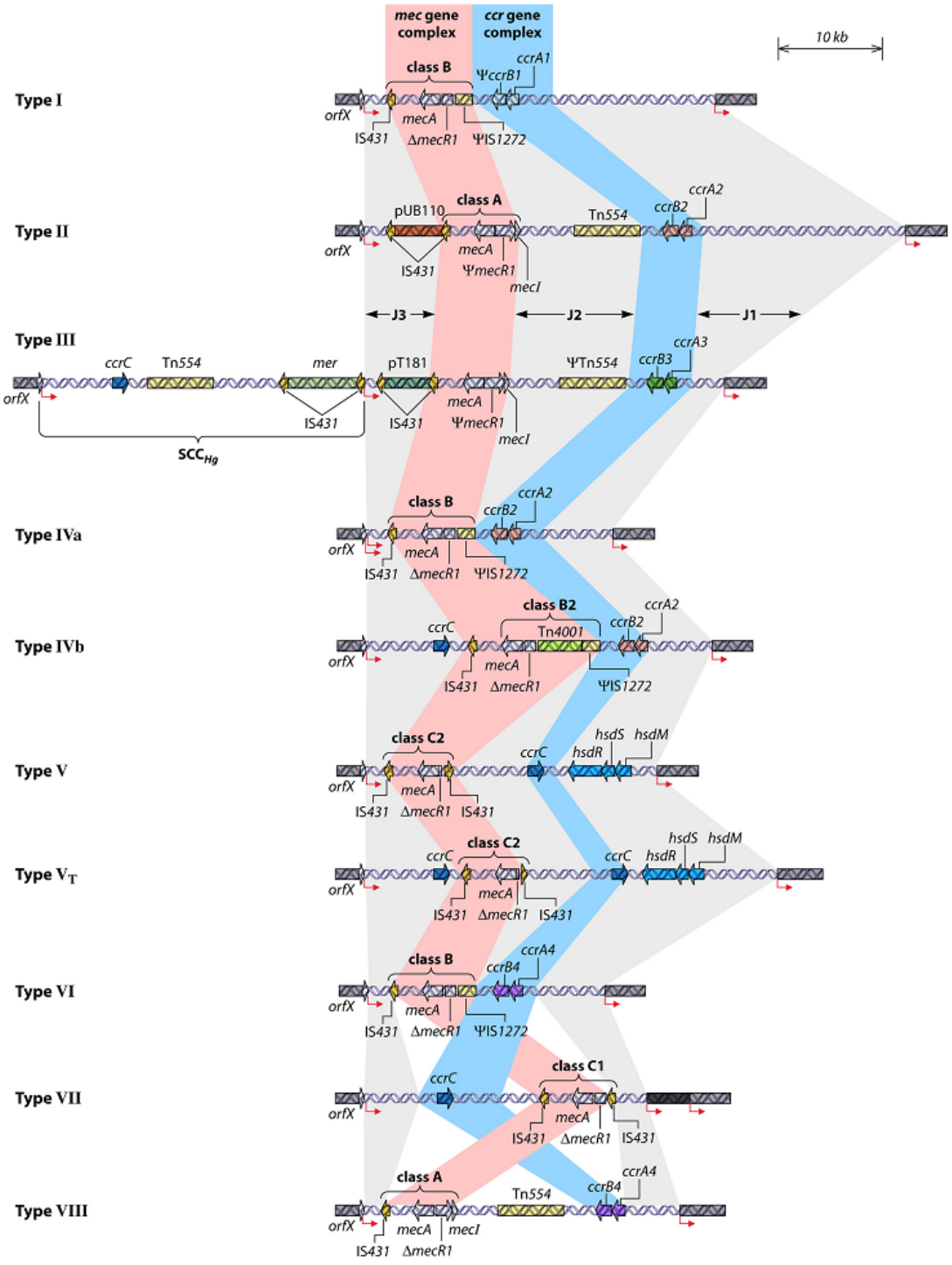

Figure 1.3. Different types of SCC*mec* elements distinguished by *ccr* gene complex and *mec* complex type. (David and Daum, Clin. Microbiol. Review, 2010)

CHAPTER II

BACKGROUND RELEVANT TO THIS STUDY

Cell Wall Synthesis and Vancomycin Resistance in *Staphylococcus aureus*

The glycopeptide antibiotic vancomycin has been used to successfully treat MRSA infections. Increasing prevalence of MRSA resulted in excessive use of vancomycin thereby increasing the selective pressure, which leads to the development of resistance. The first clinical strain that was reported to be vancomycin resistant was Mu50, which was isolated in 1997 from a Japanese male patient who had been under vancomycin treatment for prolonged period of time before the strain was isolated. Subsequently, there have been reports of similar strains being isolated from other parts of the world.

The mechanism of intermediate vancomycin resistance seems novel. Many of the vancomycin non–susceptible strains tested have been found negative for *van* gene complex that confer vancomycin resistance in enterococci. Although there are phenotypic variations among the vancomycin non-susceptible strains from different parts of the world, the most common characteristic feature is a very thick cell wall. Increase in cell wall synthesis has been reported from both the clinical and laboratory-generated strains.

Vancomycin targets the D-ala-D-ala subunits of the peptidoglycan leading to cell death by inhibiting cell wall crosslinking. It is important to understand the cell wall synthesis in order to fully understand the action of vancomycin and how the bacterium resists it. Walsh et al (16) described the process as following. *S. aureus* cell wall is composed of peptidoglycan, which is a polysaccharide matrix composed of unbranched

β-1,4-linked chains containing alternating subunits of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNac). Biosynthesis of glucosamine-1-phosphate from glucose and/or glucosamine initiates the process of peptidoglycan synthesis. Pyrimidine UDP is then linked to glucosamine-1-phosphate to produce UDP-*N*-acetyl-glucosamine (UDP-GlcNAc). A second molecule of UDP-GlcNAc combines with phosphoenol pyruvate, which is produced from glucose in glycolysis to form UDP-*N*-acetyl-muramic acid (UDP-MurNAc). An L-alanine is then linked to the UDP-MurNAc residue followed by D-glutamate, L-lysine, and D-alanyl-D-alnine to form the UDP-MurNAc pentapeptide. It is then transferred to the C55-undecaprenylphosphate to form LIPID-I of the cytoplasmic membrane peptidoglycan transporter. Next step is the formation of pentaglycine side chain on the L-lysine component. The D-glutamate component is then amidated to D-glutamine using an L-glutamine, which acts as a NH_4^+ donor. This complex is then transferred to the outside of the cell. It is then joined to the nascent peptidoglycan chain by the activity of transglycosylase enzymes, which links the moieties Glc-NAc-MurNAc –pentapeptide-pentaglycine. Transpeptidase enzymes then join the pentaglycine chain of one monomer to the penultimate D-Ala residue of the pentapeptide of the next peptidoglycan chain, releasing the last D-Ala residue. This cleavage of the last D-Ala residue is a key step to the cell wall crosslinking process. Although theoretically all D-Ala-D-Ala residues should be digested in the process, approximately 20% of them remain intact. Vancomycin binds to the D-Ala-D-ala residue of the peptidoglycan precursor UDP-MurNAc-pentapeptide and inhibit transpeptidation and nascent peptidoglycan synthesis. Bacterium dies because of incomplete cell wall.

Vancomycin non-susceptible strains have been found to possess an increased cell wall synthesis coupled with reduced crosslinks in the peptidoglycan. It is proposed that reduced levels of peptidoglycan crosslinking leads to an increased amount of intact D-Ala-D-ala side chains to which vancomycin can bind. The main location of cell wall synthesis in *S. aureus* is the tip of the division septum of a bacterium and not the entire plasma membrane surface (17). Therefore, in order for vancomycin to prevent cell wall synthesis, it would have to diffuse through the cell wall to the tip of the division septum, which is approximately a 25-nm path (18). The D-Ala-D-Ala-vancomycin complex in the outer edges of the peptidoglycan are likely to sterically hinder the other vancomycin molecules preventing its diffusion from the environment to the cell's active site, which is the tip of the division septum in the cytoplasmic membrane (19, 20). This enables vancomycin non-susceptible strains to continue synthesizing peptidoglycan and to survive in a vancomycin-containing environment.

Stress response regulator genes, such as sigma B, have been found to regulate antibiotic resistance in *S. aureus.* Cold shock is one of the stress factors that bacteria may encounter. Designated cold shock proteins (CSPs) respond to the environment and induce the expression of necessary genes to keep bacterial cells alive. MsaB protein (previously called Cold shock protein A) of *S. aureus* is predicted to contain a cold shock DNA binding domain (CSD) and bears homology to *E. coli* and *B. subtilis* cold shock proteins (CSPs). Bacteria respond to abrupt decrease in temperature by changing the protein expression patterns (21). Although expression of most cellular proteins decrease during the cold stress, expression of cold shock proteins reaches its peak at this time. One of the first proteins described to express under cold shock is the *E. coli* cold shock protein CspA

(22). Although most CSPs are found to be expressed during cold shock, CspE and CspC are found to express constitutively at normal growth conditions at 37°C. Moreover, the constitutively expressed CSPs were thought to be regulating two major stress response proteins of *E. coli* RpoS and UspA (23). In the last chapter of my dissertation, I investigated the role of *msaB* gene and MsaB protein in survival of *S. aureus* in stress environment.

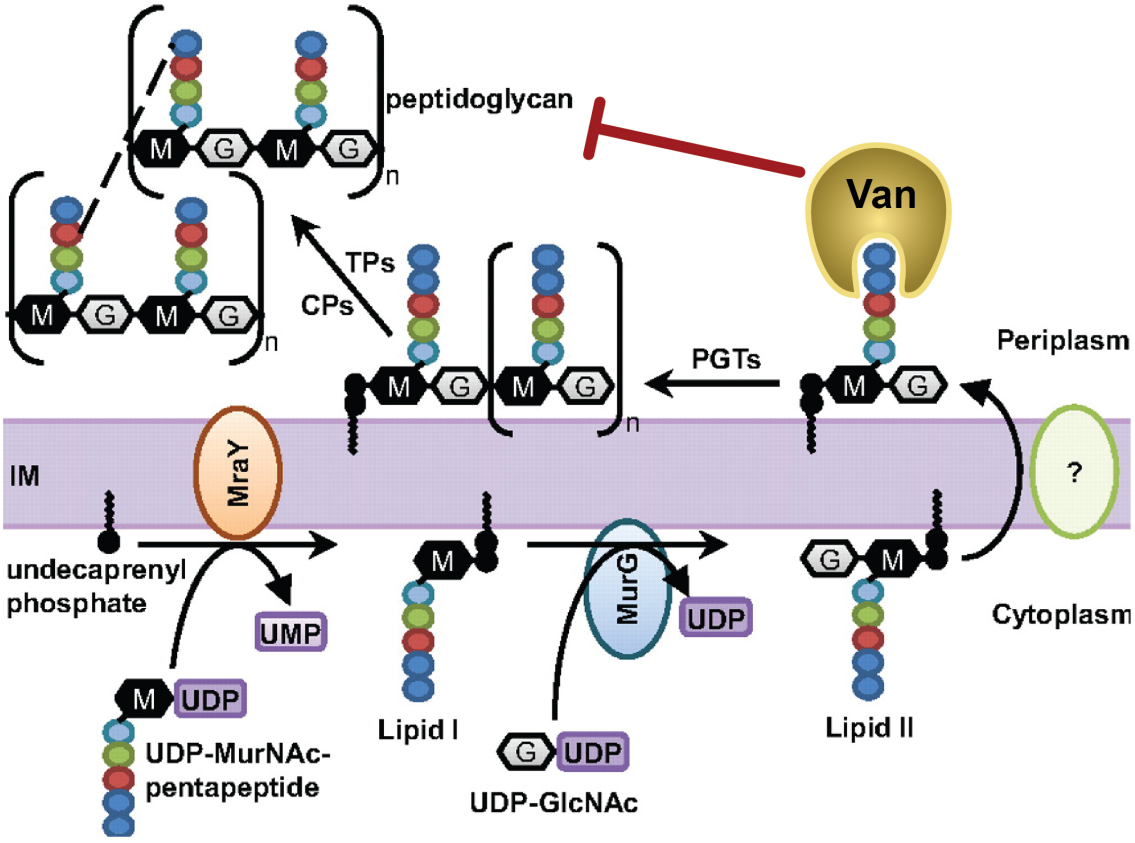

Figure 2.1. Model of mechanism of action of vancomycin that binds to the lipid II of peptidoglycan precursor and blocks the downstream pathway of cell-wall biosynthesis. (Figure courtesy: Natividad Ruiz, PNAS 2008)

CHAPTER III

THE *msaABCR* OPERON REGULATES RESISTANCE IN VANCOMYCIN-INTERMEDIATE *Staphylococcus aureus* STRAINS

Abstract

Vancomycin-intermediate *Staphylococcus aureus* (VISA) strains poses a serious public health threat. The molecular mechanism responsible for this resistance is not yet completely understood. In this part of my study, I investigate the role of the newly discovered *msaABCR* operon in establishing vancomycin resistance in three clinical VISA strains, Mu50, HIP6297, and LIM2. Deletion of the *msaABCR* operon resulted in a significant decrease in the vancomycin MIC (from 6.25 to 1.56 µg/mL) and a significant reduction of cell wall thickness in all three VISA strains. Mutants exhibited a significantly slower growth in medium containing vancomycin, at a concentration greater than 2 µg/ml, compared with the wild-type strain. Mutants also revealed significantly less capability to bind vancomycin molecules in medium compared to wild type. From this study, I conclude that the *msaABCR* operon in *S. aureus* strains contributes to resistance against vancomycin and cell wall synthesis.

Introduction

Staphylococcus aureus is an important public health pathogen in both community and healthcare settings. *S. aureus* can cause wide range of infections that can be superficial (e.g., skin and soft tissue infections) or life threatening (e.g., toxic shock, endocarditis, and septicemia) (24). One of the key attributes of *S. aureus* as a pathogen is the acquisition of antibiotic resistance, as evidenced by the rise and widespread prevalence of methicillin-resistant *S. aureus* strains (MRSA). The recent emergence of

community-acquired MRSA strains that are more virulent compared to other strains has worsened the situation (25, 26). Indeed, since the 1990s, MRSA strains have been responsible for one third of all *S. aureus* infections worldwide (27). Vancomycin, a glycopeptide antibiotic has been the drug of choice to treat MRSA infections for a long time (24). However, vancomycin non-susceptibility in *S. aureus* is becoming increasingly prevalent.

Vancomycin-resistant *S. aureus* strains have been categorized into two major groups depending on their level of resistance, as measured by the minimum inhibitory concentration (MIC). According to the Clinical Laboratory Standards Institute (CLSI), *S. aureus* strains are considered sensitive if their MIC of vancomycin is less than or equal to 2 μg/ml. Strains are called vancomycin-intermediate *S. aureus* (VISA) if their MIC ranges from 4 to 8 μg/ml, while strains that show a vancomycin MIC higher than 16 μg/ml are classified as vancomycin-resistant *S. aureus* (VRSA). The prevalence of the VRSA strains is very low compared to VISA strains, which are more frequently encountered (28)

First report of VISA strains came from a hospital in Japan in 1997 and since then these strains have emerged as a serious threat to public health owing to the extensive use of vancomycin in healthcare settings (29). Since then, several MRSA strains with intermediate-level resistance to vancomycin have been isolated from all over the world (30-38). In clinical settings, patients infected by VISA strains tend to have several co-morbidities like diabetes mellitus, end-stage renal disease, or cancer; and usually have been found to have received a prolonged course of vancomycin prior to isolation and

detection of VISA. Additionally, infections also can occur in biomedical devices such as catheters and transplants and they do not respond well to vancomycin treatment (16).

Despite a number of studies performed by several people, the mechanism of vancomycin intermediate resistance is not completely understood. As mentioned before, vancomycin targets the cell wall and inhibits its synthesis by attaching to D-Ala-D-Ala residues of pentapeptide sidechain of undecaprenylphosphate-MurNAc-pentapeptide. Multiple genetic and biochemical mechanisms have been found to play together to establish the VISA phenotypes. These include: a thickened cell wall (39), reduced autolytic activity (40), increased proportion of non-amidated muropeptides (41), and reduced cross-linking between murein monomers caused by down-regulation of penicillin-binding protein 4 (PBP4) (42, 43), which ultimately leads to an accumulation of D-Ala-D-Ala pseudo-targets in the cell wall (20). Moreover, several genes have been found to be altered in expression in VISA strains. These include genes like *atl* (major autolysin), *lytM* (peptidoglycan hydrolase) (40), *sceD* (transglycosylase) (44), *mprF* (lysyl-phosphatidyl glycerol) (45), *dltA* (teichoic acid biosynthesis regulator), *sigB* (46-49), *ddh* (50, 51), *tcaA* (52), and two-component systems *graSR*, *vraSR*, and *walKR* (53). Yet, we do not fully understand how *S. aureus* strains avert the effects of vancomycin. However, from all these studies, it is clear that resistance to vancomycin is significantly more complex than what we have thought about it so far.

Previously, our lab has identified and characterized the *msaABCR* operon and shown that it positively regulates expression of a global regulator gene *sarA*, development of biofilm, and virulence of *S. aureus* strains(54-56). The *msaABCR* operon is a four gene operon consisting of *msaA* (SA1235), *msaB* (SA1234), *msaC* (SA1233)

which are all in same orientation, and newly discovered *msaR* antisense RNA which is in opposite orientation (56). One of the most important phenotypes observed in the *msaABCR* operon mutant was increased triton-X–induced autolysis rate (Figure 3.1). A reduced rate of autolysis has been previously correlated with vancomycin-intermediate resistance (40, 57). Although the mechanism unclear, some studies have suggested that reduced autolysis indeed leads to resistance against multiple cell-wall active antibiotics (58). Also, inactivation of the *sarA* has been shown to lead to a decrease in the vancomycin MIC of laboratory-derived VISA strains (59). These findings led me to investigate the role of the *msaABCR* operon in the establishment of vancomycin intermediate resistance phenotype of *S. aureus,* and indeed our results show that the *msaABCR* operon contributes significantly vancomycin resistance in three different clinical VISA strains, Mu50, HIP6297 and LIM2.

Materials and Methods

Bacterial strains and culture conditions

Wild type and mutant strains of *S. aureus* are listed in Table 3.1. *Escherichia coli* strain TOP10 (Life Technologies, New York, USA) was used for molecular cloning experiments. Luria-Bertani medium was used for *E. coli* culture. Brain-heart infusion (BHI) broth and agar were routinely used for culturing all VISA strains unless otherwise mentioned. Tryptic soy broth and agar were used for culturing *S. aureus* strain RN4220. For maintaining plasmids, appropriate antibiotics were added to the media at a concentration of 100 µg/mL of ampicillin for *E. coli* and 10 µg/mL of chloramphenicol for *S. aureus.*

Generation of the deletion mutants and complementation

All plasmids and primers used in this study are listed in Table 3.2. Isogenic deletion mutants of the *msaABCR* operon were generated in the VISA Mu50, HIP6297, and LIM2 strains using the plasmid pKOR1, as described previously (60). Briefly, approximately 1 kb of sequence up- and downstream of the operon region was PCR amplified with operon deletion primers (Operon del 1 through 4). The PCR products were digested with restriction enzyme *Bam*HI and ligated. The ligation product was amplified again with the terminal AttB-containing operon deletion primers (Operon del 1 and 4) and recombined into pKOR1 using the BP Clonase Kit (Life Technologies) resulting in pKOR1Δ*msaABCR*. This plasmid was introduced into *S. aureus* RN4220 by electroporation. The constructs were subsequently transduced into strains Mu50, HIP6297 and LIM2 using bacteriophage φ. Mutagenesis was performed using the method described by Bae and Schneewind, 2006 (60). The mutation was confirmed by PCR using terminal primers.

For trans-complementation, a low copy number plasmid pCN34 was used (61). To replace the kanamycin (*aphA-3*) selection marker of pCN34 with a chloramphenicol marker, the *cat* gene was PCR amplified from pHV1249 (62) using the primers Cm-F and Cm-R to generate an *Apa*I–*Xho*I fragment, which was then cloned into pCN34 resulting in plasmid pCN34(Cm^r). A 1.7-kb *Bam*HI and *Eco*RI fragment was then PCR amplified using the primers Comp-F1 and Comp-R1 from the genomic DNA of *S. aureus* strain Mu50/HIP6297/LIM2 and cloned into pCN34(Cm^r) generating the pCN34(Cm^r)–*msaABCR* operon. This plasmid was introduced into *S. aureus* strain RN4220 by electroporation and subsequently transduced into the deletion mutant strains.

Complemented strains were always maintained in media containing 10 µg/mL of chloramphenicol.

Triton X-100 induced autolysis

Autolysis assay was performed as described by Mani et al, 1993 (63). Briefly, overnight cultures were normalized to $OD_{600} = 0.05$ in BHI containing 1M NaCl. Cells were harvested when they reach $OD_{600} = 0.7$, resuspended in autolysis buffer (Tris-HCl pH=7.5, 0.05% triton X-100) and incubated in 37°C. OD_{600} was recorded each hour.

Vancomycin susceptibility assay

Antimicrobial susceptibility of all strains was measured in triplicate by the broth microdilution method according to the CLSI guidelines (64). Mueller-Hinton broth supplemented with 2% NaCl (CSMHB) was used for all broth microdilution experiments. Antimicrobial containing wells were inoculated with 5×10^5 CFU/mL (65). After overnight incubation at 35°C, wells were analyzed for visible bacterial growth as exhibited by turbidity. The lowest concentration of vancomycin that prevented bacterial growth was considered to be the MIC.

Growth curve

An aliquot of fresh bacterial culture was diluted to about 1×10^5 CFU/mL in 25 mL of fresh BHI broth and grown at 37°C with shaking. The optical density was measured at 600 nm at time intervals. To analyze the growth defect of the *msaABCR* operon mutants in vancomycin containing media, BHI supplemented with vancomycin at a concentration of 1, 2, and 3 µg/mL was used. For replenishment of the medium in experiments plotting prolonged growth curves, vancomycin at a concentration of 4 µg/mL was added every 6 h.

Population analysis profile

Population analysis helps to determine how many cells within a given number of cells are resistant to a range of concentrations of a particular antibiotic (39). Overnight cultures of *S. aureus* strains Mu50, HIP6297, and their respective mutant strains were diluted to 10^{-4} and 10^{-6} and 100 µL of each dilution were plated onto BHI agar plates containing various concentration of vancomycin. Plates were incubated for 48 h at 37°C, and CFUs were plotted in a logarithmic scale against the vancomycin concentration.

Transmission electron microscopy

Preparation and examination of *S. aureus* cells by transmission electron microscopy were performed by the method described previously (66). Briefly, exponentially growing cells were fixed in 2% glutaraldehyde in 0.1 M sodium phosphate buffer and treated with 1% osmium tetroxide for 2 h at 4°C. Cells were dehydrated, and embedded in low viscosity embedding media (Electron Microscopy Sciences). Ultra-thin sections were stained with uranyl acetate and lead acetate. Cell wall thicknesses were measured using photographic images at a ×30,000 final magnification. Fifteen cells of each strain with approximately equatorial cut surfaces were measured and results were expressed as the mean value ± standard deviation (SD). Statistical analysis was performed using the MedCalc software (MedCalc V13, www.medcalc.org). P value less than 0.05 was considered significant.

Vancomycin binding assay

The degree of binding of vancomycin molecules to the cell wall of wild-type strains and their respective *msaABCR* operon deletion mutants was determined using a dot blotting technique. Specifically, overnight cultures were diluted to $OD_{600} = 0.05$ in

BHI medium containing no antibiotics. When the cells reached the mid log phase, vancomycin was added at a concentration of 4 μg/mL to the culture. After 1 h, 500 μL of culture was collected and centrifuged to pellet the cells. Then, 10 μL of the supernatant was loaded onto a nitrocellulose membrane and air-dried. The membrane was then blocked with non-fat skimmed milk, incubated with an anti-vancomycin primary antibody, HRP-conjugated secondary antibody, and developed with SuperSignal® West Pico Chemiluminescent substrate (Thermo Scientific, Rockford, Illinois, USA). The intensity of the signal was measured using the ImageJ software (67)

RNA extraction, reverse transcription, and qRT-PCR

An aliquot of an overnight culture was normalized to OD_{600} = 0.05 and grown to exponential phase. At this time, a dose of 4 μg/mL of vancomycin was added to the culture. A control culture was maintained where no vancomycin was added ("none" condition). Cells were grown for 1 h and harvested by centrifugation. The bacterial pellet was treated with RNAprotect™ Bacteria Reagent (Qiagen, Valencia, CA, USA) and stored at −80°C. RNA from the pellet was isolated using the RNeasy® mini kit (Qiagen) and dissolved in DEPC-treated H_2O. RNA quality was analyzed by determining the $A_{260/280}$ ratio using a nanodrop spectrophotometer (Thermo). Reverse transcription was carried out from 1000 ng of RNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. All real-time PCRs were performed in triplicate. The primers used for real-time PCR are listed in Table 3.2. The fold change in gene expression was calculated using the formula $2^{-\Delta\Delta ct}$ (68), using the *gyrA* gene as an internal control. Fold change values were statistically analyzed by the independent sample t test using MedCalc software.

Absolute quantification of the sigB transcript

Absolute quantification of the *sigB* transcript was performed by the method previously described by Chini *et al*. 2007 (69). Briefly, the *sigB* and *gyrA* genes were amplified from chromosomal DNA using primers external to the primers used for qRT-PCR. These amplicons were purified, and their concentrations were measured using a nanodrop spectrophotometer. The corresponding concentration was converted to copies per microliter by a previously described method (70). Tenfold serial dilutions (range 10^2–10^6) of these amplicons were used as templates for qRT-PCR. Standard curves were generated by plotting C_t values against the log of the copy numbers (Log Starting Quantity, SQ). Starting quantities of "unknown" samples (cDNA of wild type and mutant) were calculated by plotting their respective C_t values on the standard curve. Copy numbers were measured by raising 10 to the power of the SQ (10^{SQ}). Copy numbers of *sigB* were normalized to that of *gyrA* and plotted. The process was repeated in triplicate independently.

Membrane purification and PBP detection

For the detection of PBPs, membranes were prepared from wild-type and *msaABCR* mutant strains following the method described by Sieradzki *et al*. 1999 (36), with some modifications. Briefly, cells were grown to the late exponential phase, harvested, and washed once with wash buffer (50 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, pH 7.5), resuspended in the same buffer with 0.5 mM phenylmethylsulfonyl fluoride and 10 mM β-mercaptoethanol. Cells were then treated with lysostaphin (100 µg/mL), DNase (20 µg/mL), and RNase (10 µg/mL) for 30 min on ice followed by sonication. Broken cells along with membranes were harvested by centrifugation at 110,000 × *g* for 40 min

at 4°C, and washed with 50 mM phosphate buffer, pH 7. Membranes were solubilized in 2% Triton X-100. Protein concentrations were measured using the BCA protein assay kit (Pierce) and a standard curve prepared from serial dilutions of bovine serum albumin.

PBPs were detected using a method described by Atilano *et al*. 2010 (71), with some modifications. Briefly, 10 µg of membrane protein were mixed with 100 µM of Bocillin-FL (Life Technologies) and incubated for 10 min at 30°C. The reaction was stopped by the addition of 5× SDS-PAGE sample buffer. Samples were separated on a 4–20% gradient gel and labeled proteins were detected using a Bio-Rad Versadoc. The gel pictures were analyzed and quantified using the ImageJ software.

Results and Discussion

Deletion of the msaABCR operon increased susceptibility to vancomycin in three VISA strains

Several studies have demonstrated that global regulators such as *sarA* and *sigB* are involved in vancomycin resistance (59, 72); however, the mechanism of regulation is not yet understood (47, 73). Because *msaABCR* is a positive regulator of *sarA* and a negative regulator of autolysis, and as mentioned earlier, both the expression of *sarA* and a decreased rate of autolysis have been shown to be important for the VISA phenotype, we attempted to understand the effect of the *msaABCR* operon on vancomycin resistance in VISA strains.

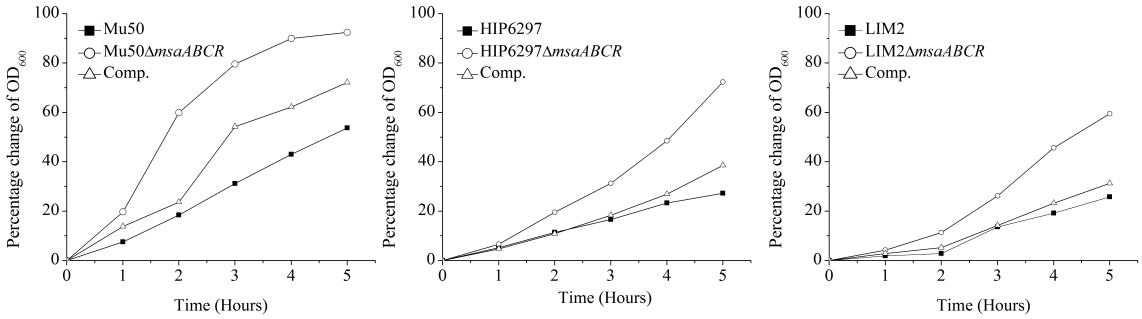

Figure 3.1. Triton X-100 induced autolysis assay shows *msaABCR* mutants lyse in a faster rate than the respective wild types

We deleted the *msaABCR* operon in three different clinical VISA strains, Mu50 (SCCmec II, ST 5) and HIP6297 (SCCmec II, ST5) and LIM2 (SCCmec I, ST247). Mu50 is a clinical VISA strain isolated in 1997 from pus of a Japanese male child with a post-surgery infection who was unresponsive to vancomycin treatment (29). This isolate has a vancomycin MIC of 8 μg/mL (29), a thick cell wall relative to its sensitive parent strain (41), reduced autolytic rates (40), reduced cell-wall cross-linking (74), and an increased number of penta-peptide side chains (41). HIP6297 is also a clinical VISA strain that was isolated from the blood of a 79-year-old male patient who had renal disease and bacteremia at a hospital in New York, USA (36). It has a vancomycin MIC of 8 μg/mL and a thick cell wall (36). LIM2, another clinical VISA strain was isolated from a 2-year old girl, in a hospital in France, who had an MRSA infection but was unresponsive to vancomycin treatment (75).

Deletion of the *msaABCR* operon resulted in a significant decrease in vancomycin resistance in all three VISA strains as showed by their MICs. Specifically, the MIC of all three strains reduced from an intermediate resistance level of 6.25 μg/mL to a susceptible level of 1.56 μg/mL (Table 3.3). Trans-complementation with the *msaABCR* operon

cloned in pCN34 plasmid into the deletion mutants, caused an increase in MIC to 3.125 μg/mL confirming that the *msaABCR* operon was indeed responsible for the decrease in their MIC (Table 3.3). It is noteworthy here that, although the MIC of the complemented strains was not exactly in the wild type VISA range (4–8 μg/mL vancomycin), still it was higher than the sensitive range which is 2–4 μg/mL of vancomycin. A vancomycin sensitive strain N315, which is genetically related to strain Mu50 (76) was used as a control in this study. As expected, deletion of the *msaABCR* operon in N315 did not further reduce the vancomycin MIC. These results, taken together, show that the *msaABCR* operon is essential for vancomycin resistance in VISA strains of different genetic backgrounds.

One of the hallmarks of VISA strains is the significant change in the growth rate relative to their sensitive counterparts (41). We measured the growth rates of the deletion mutants at different vancomycin concentrations (1, 2, and 3 μg/mL). We found that wild-type strains displayed a steady growth rate and reached a stationary phase after 6 h at all three concentrations of vancomycin, whereas the three mutant strains did not show any growth after 6 h at a vancomycin concentration of 2 μg/mL or higher (Figure 3.2). Interestingly, all the mutants showed growth after a 20-h lag period unless the medium was replenished with vancomycin (Figure 3.3). This observation supports previous findings by Cui *et al*. 2000 (41), and confirms the increased susceptibility of the *msaABCR* operon mutant to vancomycin.

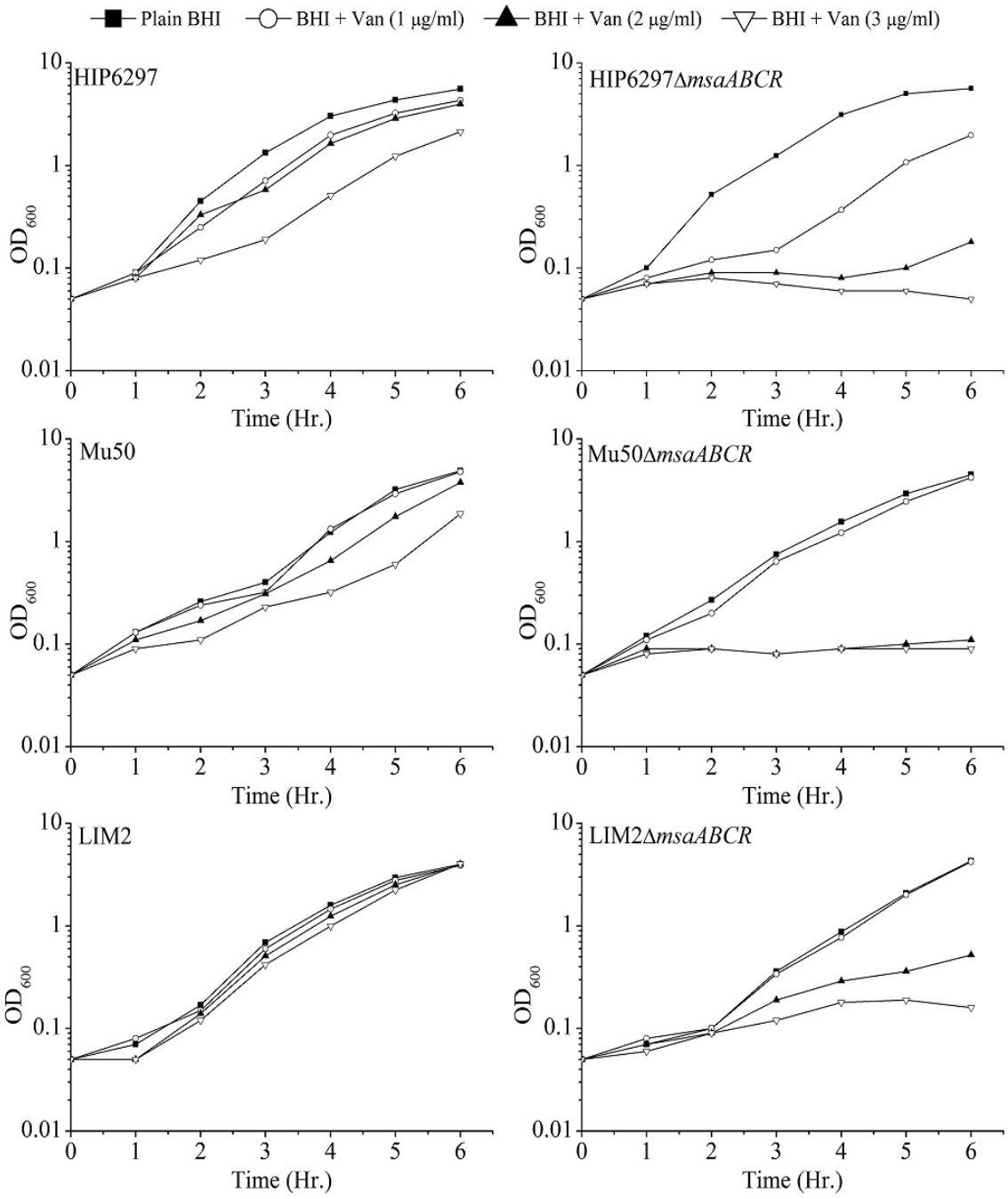

Figure 3.2. Growth curves of wild types Mu50, HIP6297 and LIM2 and their corresponding *msaABCR* deletion mutants in plain media and in different concentrations of vancomycin (1, 2, and 3 μg/ml). Axes are converted to log scales.

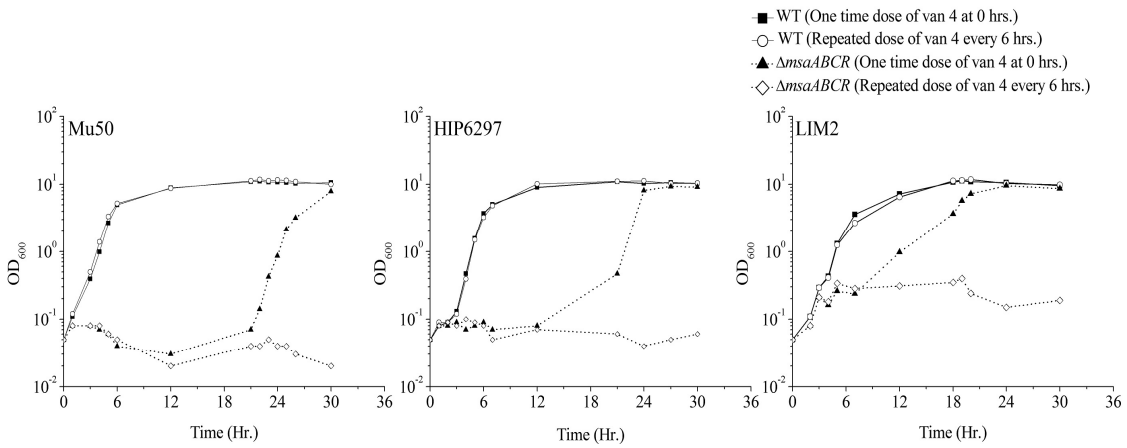

Figure 3.3. An elongated growth curve of wild type Mu50, HIP6297, and LIM2 and their respective *msaABCR* operon deletion mutants in medium containing 4 μg/ml vancomycin or medium replenished with 4 μg/ml vancomycin every 6 hours.

To further characterize the population of the deletion mutants with regard to vancomycin resistance, we evaluated the homogeneity of the cells using a population analysis profile (Figure 3.4) (39). With wild-type strain Mu50, 10^{10} cells/mL were resistant to vancomycin at concentrations up to 3 μg/mL and 10^{7} cells/mL were resistant to a vancomycin concentration of 4 μg/mL. This indicated a small degree of heterogeneity in resistance. However, with the Mu50 *msaABCR* operon mutant, 10^{9} cells/mL were resistant to a vancomycin concentration of 1 μg/mL but no cells were resistant to a vancomycin concentration higher than 1 μg/mL. This indicated that the *msaABCR* operon mutant had a homogenously sensitive population structure with regard to vancomycin. Likewise, in the case of HIP6297, 10^{10} cells/mL were resistant to 4 μg/mL of vancomycin and 10^{9} cells/mL were resistant to 5 μg/mL of vancomycin. The *msaABCR* operon mutant however possessed 10^{9} resistant cells per mL in the presence of 3 μg/mL vancomycin and no resistant cells at higher concentrations. Wild type LIM2 maintained 10^{10} cells/ml up to 5 μg/mL vancomycin, whereas its *msaABCR* mutant maintained 10^{8} cells/ml only up to 2 μg/mL vancomycin. These results indicated that

mutation of the *msaABCR* operon led to a population of cells that are homogenously sensitive to vancomycin. These results also suggested that the *msaABCR* operon is a regulator of resistance to vancomycin. Population analysis revealed that both of the mutants are homogenously sensitive to vancomycin. Indeed, the *msaABCR* deletion mutants did not show any resistant subpopulations in the presence of vancomycin at concentrations greater than 3 μg/mL.

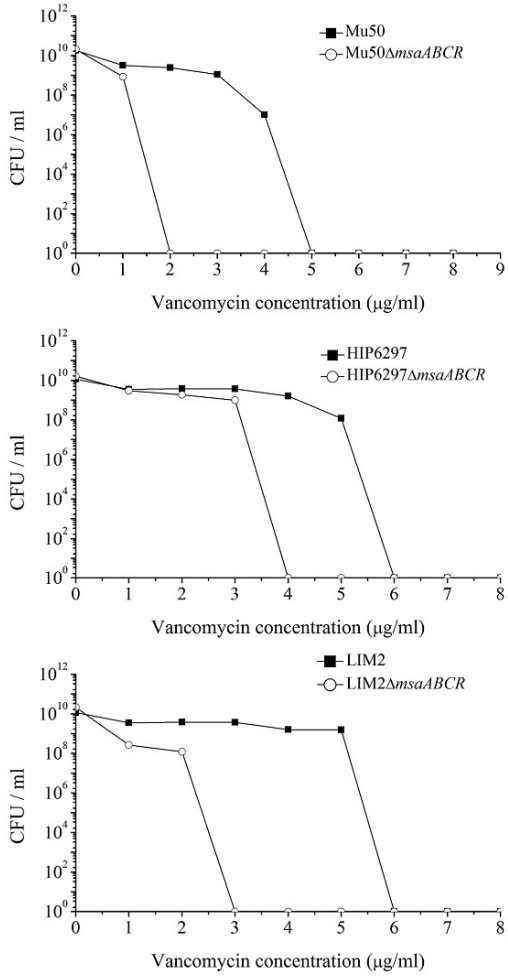

Figure 3.4. Population Analysis Profile of the wild types Mu50, HIP6297 and LIM2 and their corresponding *msaABCR* operon deletion mutants.

This phenotype is of significance to the clinical treatment of VISA infections because some VISA strains, such as Mu50, have been shown to maintain a resistant subpopulation

to vancomycin. Indeed, Cui *et al*. 2003 (39) demonstrated that passage of strain Mu50 through a drug-free medium (35 days) led to a drop in the vancomycin MIC from 8 μg/mL to 2 μg/mL. However, population analysis of the passage-derived strain revealed a resistant subpopulation that was able to grow in the presence of up to 8 μg/mL of vancomycin (39). Such VISA strains have been defined as hetero-VISA (h-VISA) and present a treatment challenge. They are difficult to treat because of the rise of resistant subpopulations during treatment with vancomycin. For instance, the hetero-VISA strain Mu3, that is isogenic to Mu50, was isolated from a Japanese male patient who was unresponsive to vancomycin treatment. While Mu3 was initially characterized as susceptible to vancomycin (MIC = 2 μg/mL) according to CLSI criteria (77), when it was grown in the presence of a high concentration of vancomycin (2–9 μg/mL), it produced resistant subclones with a MIC of 6–8 μg/mL (comparable to Mu50) at a frequency of 1 in 10^6. This is relevant to patients infected with h-VISA strains, where peak tissue concentrations of vancomycin can be from 2 to 5 μg/mL depending on the dosage and frequency of treatment (78). These studies show that the rise of a resistant subpopulation is likely; however, the mechanism that leads to this is not yet understood. The absence of resistant subpopulations in the *msaABCR* operon deletion mutants is therefore very interesting and suggests a role of the operon in this process. Targeting regulators such as the *msaABCR* operon, which eliminate h-VISA, could therefore be potentially beneficial in developing new treatments for VISA infections.

Previously, *sarA* and *sigB* inactivation in laboratory-derived vancomycin resistant/intermediate strains also resulted in a decrease in the vancomycin MIC. Singh *et al*. 2003 (72) demonstrated that a *sigB* deletion mutant of the laboratory-derived strain

13136$p^{-}m^{+}V_{20}$ had a vancomycin MIC of 4–6 μg/mL after population analysis compared with the wild-type strain, which had a vancomycin MIC of 12–14 μg/mL. The population analysis profile of the mutant strain displayed a homogenously sensitive population structure. Similarly, in the study by Lamichane-Khadka *et al*. 2009 (40), the *sarA* insertion mutant of $BB270V_{15}$ showed an MIC of 3 μg/mL in agar dilution analysis while the wild type showed a vancomycin MIC of 8 μg/mL. In the population analysis profile, the mutant displayed a homogenously sensitive population structure. These findings support our hypothesis that mutation of major global regulators could have a profound effect on vancomycin resistance of VISA strains and may render the strains homogenously sensitive to vancomycin, which in turn could be clinically beneficial.

The msaABCR operon regulates cell wall morphology

Vancomycin is an inhibitor of cell wall synthesis in *S. aureus* and other Gram-positive bacteria. It binds to the D-Ala-D-Ala residue of the pentapeptide sidechain of undecaprenylphosphate-MurNAc-pentapeptide, a cell wall precursor, and forms a stable non-covalent complex. As a result, this complex cannot be used in the biosynthesis of the cell wall (79). VISA strains, including Mu50 and HIP6297, have thick cell walls relative to their sensitive counterparts (36, 39, 41, 66, 80, 81). Cui *et al*. 2003 (39) showed that cell wall thickening is the most consistent feature of vancomycin intermediate and resistant strains. They also showed a positive correlation between the thickness of the cell wall and vancomycin MIC in a study of 48 *S. aureus* strains including VISA and VRSA strains.

We examined cell wall thickness of the *msaABCR* operon mutants using transmission electron microscopy. Both mutants showed a significant decrease (50%) in

cell wall thickness and a smoother texture relative to their wild-type strains (Table 3.4, Figure 3.5 a-b). These results suggest that the *msaABCR* operon plays a role in cell wall synthesis and contributes to cell wall thickness in VISA strains.

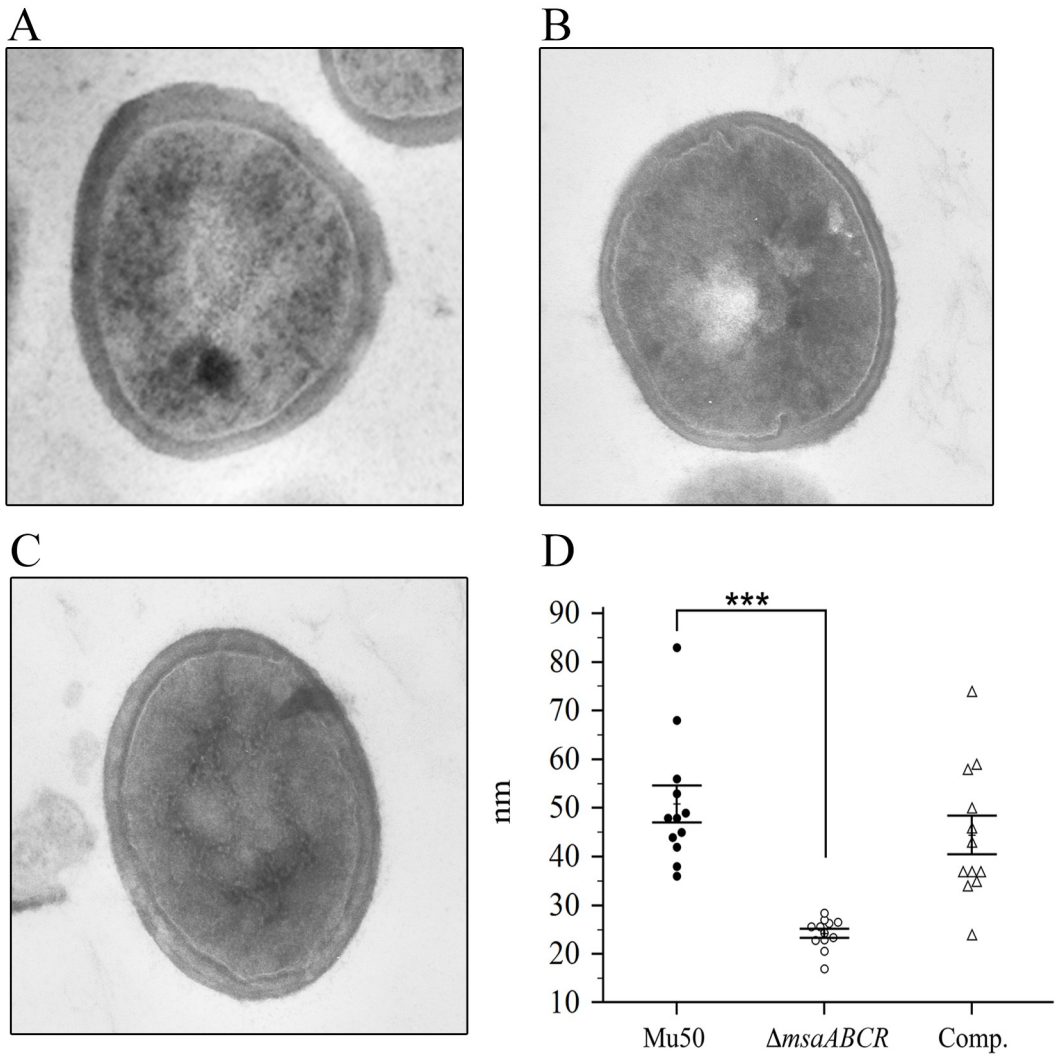

Figure 3.5(a). Transmission Electron Micrograph of cell walls of wild type Mu50 (A), Mu50Δ*msaABCR* (B), complemented mutants (C), and independent sample t-test of cell wall thicknesses of all three strains (D). nm: nanometer; ns: non-significant. ***: $p<0.0001$

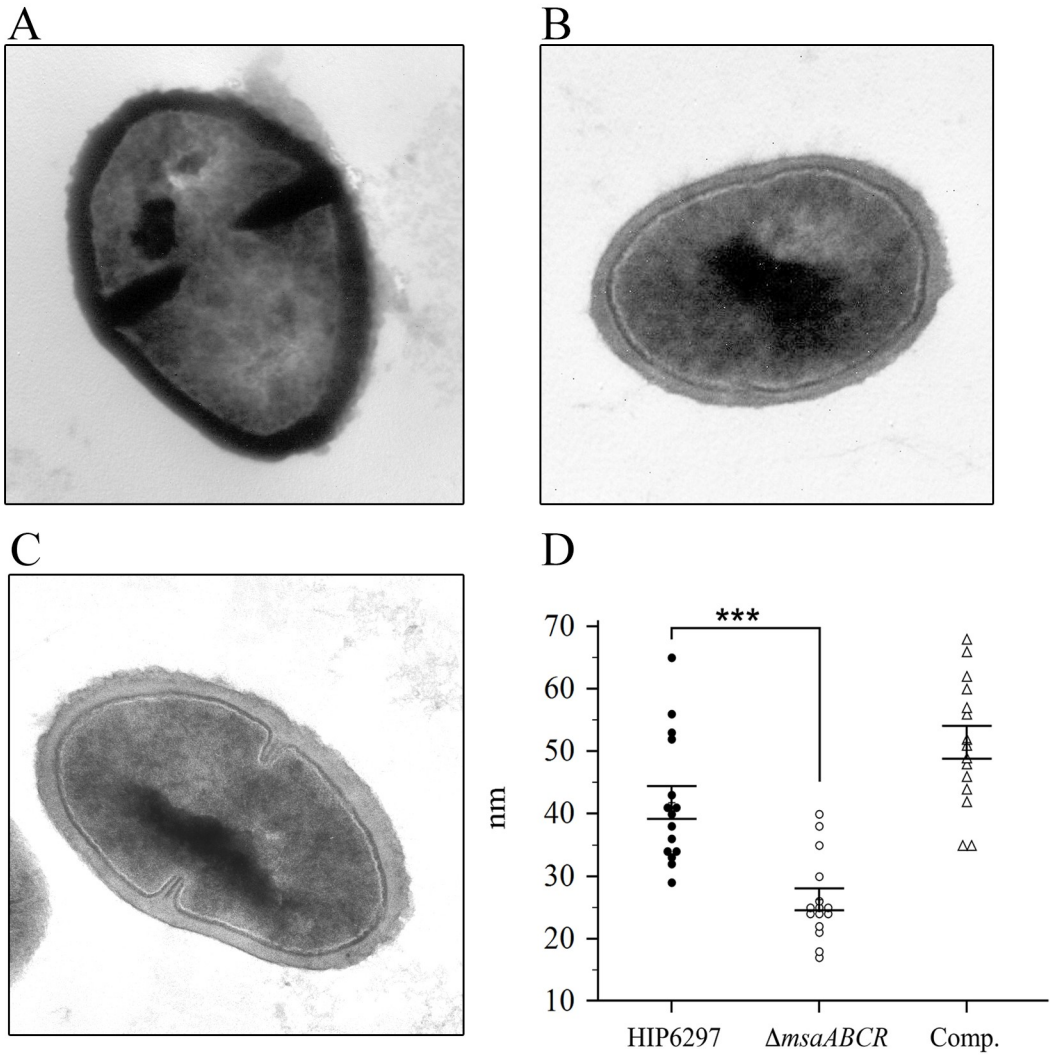

Figure 3.5(b). Transmission Electron Micrograph of cell walls of wild type HIP6297 (A), HIP6297Δ*msaABCR* (B), complemented mutants (C), and independent sample t-test of cell wall thicknesses of all three strains (D). nm: nanometer, ***: $p<0.0001$

The main location of cell wall synthesis in *S. aureus* has been shown to be the tip of the division septum of a bacterium and not the entire plasma membrane surface (17). Therefore, for vancomycin to prevent cell wall synthesis, it would have to diffuse through the cell wall to the tip of the division septum, which is approximately a 25-nm path (18). Pfeltz *et al*. (2000) proposed that a thickened cell wall increases the path that vancomycin has to travel, preventing the diffusion of vancomycin molecules from the environment to

the cell's active site, which is the tip of the division septum in the cytoplasmic membrane (19, 20). This enables VISA and VRSA strains to continue synthesizing peptidoglycan and to survive in a vancomycin-containing environment. Our results suggest that the observed sensitivity of *msaABCR* operon mutants to vancomycin may be due to the decrease in cell wall thickness.

As stated earlier, one of the key phenotypes of the *msaABCR* operon mutants is the prolonged lag phase in the vancomycin-containing medium, which may also be due to the thinning of the cell wall. A study comparing VSSA with VISA strains found that the length of the lag phase negatively correlated with cell wall thickness (39, 41). It was proposed that a thin cell wall binds vancomycin molecules at a significantly slower rate than in the wild-type strains and therefore removes drug molecules from the medium at a slower rate, which in turn results in a prolonged lag phase.

Indeed, we observed a three-fold decrease in the binding of vancomycin molecules to the cell wall of the *msaABCR* mutants compared with the respective wild-type cells (Figure 3.6). To date, however, the mechanistic link between the length of the lag phase and the thickness of the cell wall has not been defined.

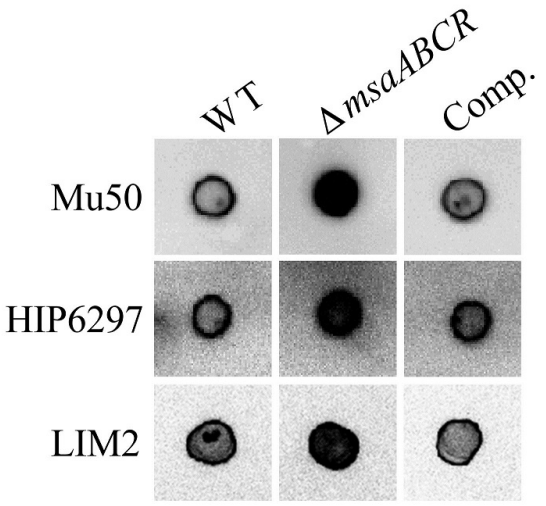

Figure 3.6. Vancomycin binding assay with wild types Mu50, HIP6297, and LIM2 and their respective Δ*msaABCR* mutants, and complemented mutants. Vancomycin was added to a growing culture of cells. After 1 hour, cells were harvested and supernatant medium was dot blotted and detected with anti-vancomycin antibody. Figure shows that Δ*msaABCR* mutants bind significantly less vancomycin than the wild types.

The msaABCR operon regulates the expression of pbp4, sigB, and mprF in a strain-dependent manner

Studies with VISA strains have yielded descriptive assessments that correlate various phenotypes with vancomycin intermediate resistance. Collectively, these studies have resulted in a set of phenotypes that are commonly observed in VISA strains. Several studies have also used a variety of approaches to identify specific genes that are associated with vancomycin resistance. These include expression studies (e.g., microarray analysis) (47, 48, 73), and comparisons of isogenic resistant and sensitive strains by targeted mutagenesis experiments (24, 45, 53, 59, 72, 82, 83). Collectively, these studies have identified the following regulator genes as critical to intermediate vancomycin resistance in *S. aureus*: *pbp4, sigB, walKR, graS, graR, mprF, dltA, vraS, vraR*, and *sarA*.

We examined the expression of these genes in the *msaABCR* operon mutants using quantitative RT-PCR in the absence and presence of vancomycin. No significant change in gene expression was observed for *graR, graS, dltA, vraSR*, and *walkR* in either of the strains. However, we found that expression of *pbp4* was increased and that of *sigB* was decreased in the Mu50 *msaABCR* mutant and expression of *mprF* was decreased in the HIP6297 *msaABCR* mutant (Figure 3.8 a-b).

The *msaABCR* mutant of Mu50, but not HIP6297, showed a three-fold increase in *pbp4* expression both in the presence and absence of vancomycin (Figure 3.8 a). We confirmed the increase in the expression of PBP4 in the Mu50 *msaABCR* operon mutant by performing a fluorescent penicillin-binding assay with Bocillin-FL to measure the levels of PBP4 protein. We found that the Mu50 *msaABCR* operon mutant produces 6.003-fold higher levels of PBP4 compared with the wild type (Figure 3.7).

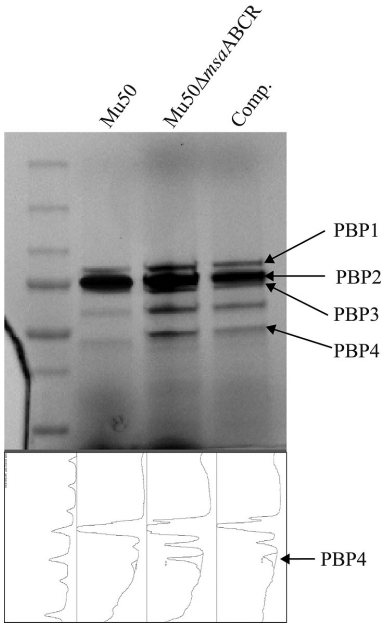

Figure 3.7. Fluorescent penicillin binding assay from purified cell membranes of wild type Mu50, Mu50Δ*msaABCR* mutant, and complemented mutants. Lower panel shows the quantification graph of the gel.

PBP4 is involved in cell wall biosynthesis as a DD-carboxypeptidase enzyme (84). PBP4 is required for cleavage of the peptide bond of D-Ala-D-Ala residue of the pentapeptide sidechain of undecaprenylphosphate-MurNAc-pentapeptide prior to cross-linking between the penultimate alanine and the third lysine of another NAM. Because vancomycin binds to D-Ala-D-Ala residues, any vancomycin-bound residue becomes unavailable for PBP4 and therefore cannot be used for crosslinking. VISA strains produce significantly less PBP4 compared with the VSSA strains (42, 85). Deletion of *pbp4* in the VSSA strain N315 caused an increase in the vancomycin MIC, which was restored by complementing *pbp4*, suggesting that the function of *pbp4* is directly related to the vancomycin MIC (42). Although the genetic regulation of *pbp4* in VISA strains is not yet understood, it has been proposed that reduced expression of PBP4 in VISA strains results in an increase of intact D-Ala-D-Ala and consequently a build-up of uncrosslinked muropeptide monomers in the cell wall. These monomers hinder the diffusion of vancomycin molecules by attaching the drug molecules to the intact D-Ala-D-Ala termini. The attached vancomycin molecules are thought to become part of the resistance mechanism by sterically hindering additional vancomycin molecules during the diffusion process (86). These findings indicate that there is a negative correlation between the expression level of *pbp4* and resistance to vancomycin. Hence, our results suggest that the *msaABCR* operon negatively controls the expression of *pbp4* in Mu50 and the increased sensitivity of the Mu50 *msaABCR* mutant to vancomycin may be attributed to the increased PBP4 activity. Further studies are needed to define the relationship between the *msaABCR* operon, PBP4 activity, and the amount of crosslinks in the cell wall.

Gram-positive bacteria respond to stress by expressing alternative sigma factors such as sigma B (encoded by *sigB*). The presence of antibiotics, such as methicillin or vancomycin, has been shown to activate the expression of sigma B in *S. aureus*, implicating this factor in antibiotic resistance (72, 87, 88).

Expression of *sigB* was significantly reduced in the *msaABCR* mutant of the Mu50 strain compared with the wild type in both the absence (4.91-fold) and presence of vancomycin (7.15-fold). Expression of *asp23*, which is directly under the regulation of *sigB*, was also down-regulated in Mu50. Interestingly, this change in expression was not observed in strain HIP6297, indicating that this regulatory effect is strain dependent. Mutation of *sigB* has been shown to reduce vancomycin resistance in a passage-derived resistant strain of *S. aureus* (72). Conversely, activation or overexpression of *sigB* led to thickening of the cell wall and increased vancomycin resistance in VSSA strain N315 (46, 49). Cell wall inhibitor antibiotics induce a stress response in *S. aureus* cells (63). Resistant cells may have the capacity to respond to and counteract the stress more efficiently than their sensitive counterparts. We propose that the *msaABCR* operon mutant of Mu50, with decreased *sigB* expression, becomes more susceptible to stress conditions including the presence of vancomycin in the medium. However, the mechanism by which the *msaABCR* operon regulates *sigB* and the reason why this regulation occurs in strain Mu50, but not in strain HIP6297, is currently unclear.

Another regulator, which is directly under the control of the stress response system, *mprF*, was found to be significantly down-regulated in the *msaABCR* operon deletion mutant of HIP6297 but not of Mu50. MprF is a putative trans-membrane protein that mediates the biosynthesis of lysylphosphatidylglycerol (45). It is a positively charged

phospholipid that contributes to a net positive charge on the cell surface. *MprF*-deficient mutants have been shown to be sensitized to cationic antimicrobial peptides of the immune system (89, 90) and vancomycin (45). This phenotype was attributed to the fact that because MprF mediates the synthesis of a positively charged lipid, absence of it leads to the accumulation of a net negative charge on the cell surface, which attracts cationic or positively charged molecules including vancomycin. Ruzin *et al*. 2003 (45) showed that an *mprF* mutant indeed binds more vancomycin to the membrane than its corresponding wild type. This may be contributing to the increased susceptibility of the HIP6297 *msaABCR* operon deletion mutant to vancomycin.

The strain-dependent variations among VISA isolates, both in terms of phenotype and genetic mutations, are not uncommon. Reduced vancomycin sensitivity has been reported to be unstable for some isolates such as HIP5836 and Mu50 (91) but not in the laboratory-derived passage-selected VISA strains such as $BB270V_{15}$, $COLV_{10}$, and $13136p^{-}m^{+}V_{20}$ (19). Cell wall thickening has been shown in most VISA isolates but to varying degrees. For instance, the thickness of the cell wall in strains JH9 and JH14 (20) appears to be many fold higher than that observed in strain Mu50. Reduced crosslinking of the muropeptides appears to be correlated with the vancomycin MIC in many VISA strains but not all (30). Boyle-Vavra *et al*. 2001 (30) demonstrated that a vancomycin trapping hypothesis and a uniform cascade of genetic events might not explain the resistance phenotype in every VISA strain isolated. However in this study, a reorganization of the cell wall was found to be common among all VISA isolates. Non-amidated residues of glutamic acids have been shown to play a role in the resistance mechanism of some, but not all, VISA strains (74). Similarly, many genes have been

correlated with vancomycin resistance either in terms of altered expression or point mutations, but none of these have been found to be present without exception. When analyzing the results of multiple studies (24, 42, 47, 86), it appears that, although theoretically lowered expression of PBP4 could be correlated with a higher vancomycin MIC, the laboratory-derived VISA mutants do not follow this pattern.

Strain-dependent variations are also found in terms of mutations in response regulator genes. Although mutations in genes such as *vraSR, graSR, clpP*, and *walKR* have been correlated with a VISA phenotype, sequence analysis of these genes in 39 VISA isolates revealed that none of these mutations are universal among all VISA strains (24). While 27 VISA strains possessed mutations in *walK*, only a few of these mutations were at the same site in the gene. Similarly, three isolates harbored mutations in *clpP*, four isolates had mutations in *graR*, and eight isolates had *vraS* mutations, but all of these mutations were at different sites within the genes. Shoji *et al*. (2011) proposed that these differences indicate the existence of alternative pathways in different VISA isolates for the establishment of vancomycin resistance (24).

Strain-dependent variation has also been observed in the function of global regulators in *S. aureus*. Zielinska *et al*. (2011) studied the regulatory role of *sarA* on the alpha-toxin phenotype of laboratory and clinical strains of *S. aureus*, and observed that *sarA* exerts a strain-dependent effect on the transcription of RNAIII and *hla* (92). Blevins *et al*. (2002) also demonstrated a strain-dependent regulatory role for *sarA* and *agr* (93). In this context, variations between strains Mu50 and HIP6297 would be expected. For instance, Mu50 harbors one mutation in VraS (I5N) and one in GraR (N197S), HIP6297 possesses neither of these but possesses a mutation in WalK (A567D) (24). Although

Mu50 and HIP6297 strains have been described to have inactivated PBP4 (86), our results indicate that the *msaABCR* operon exerts a strain-dependent effect on *pbp4* expression.

Strain variations in VISA isolates, such as the ones in this study, have made it difficult to define a common mechanism to the establishment of the VISA phenotype. In conclusion, we have shown that the *msaABCR* operon plays a key role in intermediate vancomycin resistance and that its inactivation leads to homogeneous vancomycin sensitivity despite strain differences.

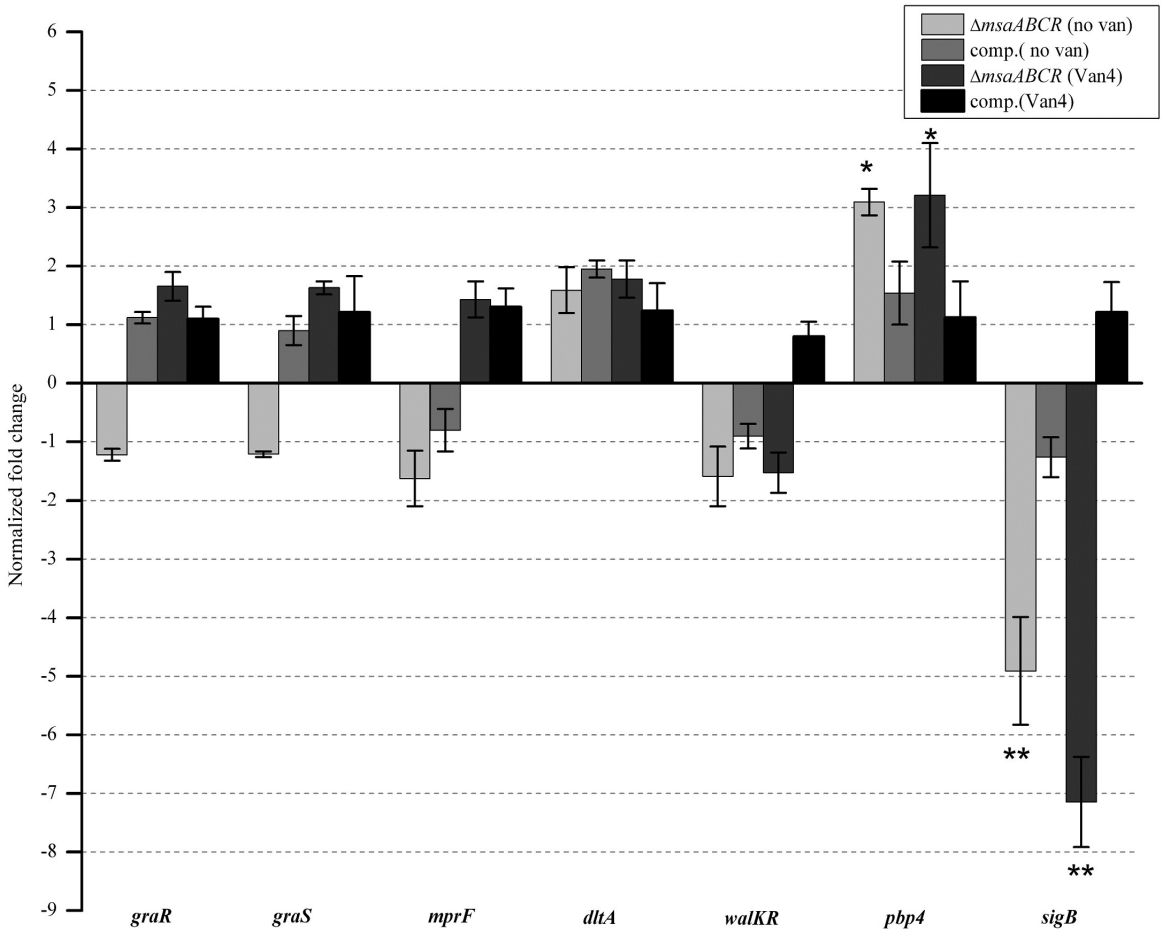

Figure 3.8(a). Transcription profile of genes linked to vancomycin resistance in Mu50Δ*msaABCR* and complemented mutant compared to wild type.

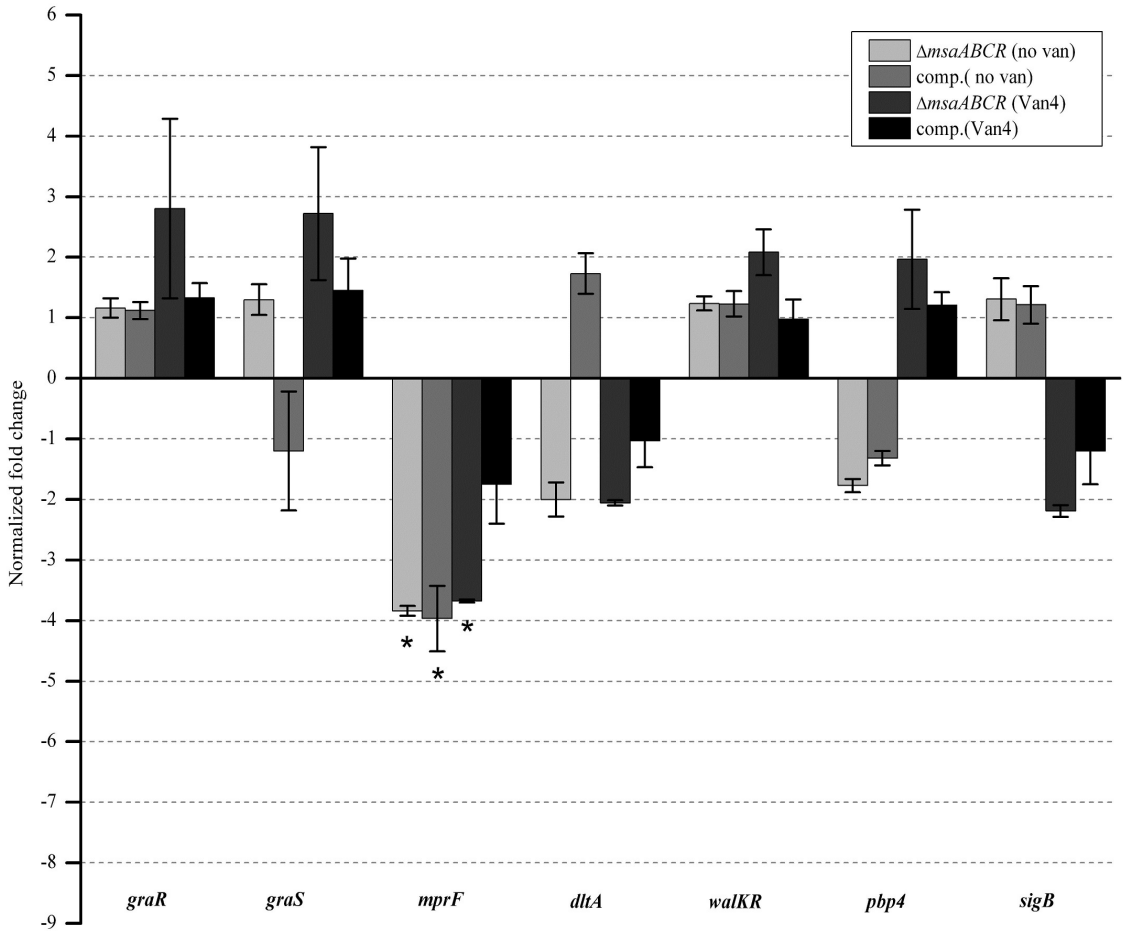

Figure 3.8(b). Transcription profile of genes linked to vancomycin resistance in HIP6297Δ*msaABCR* and complemented mutants in comparison to wild type.

Table 3.1

Strains and Plasmids used in this study

Strains or Plasmids	Relevant feature	Reference or Source
Strains		
E. coli		
TOP10	F⁻ □80*lacZ*ΔM15 *rec*A1	Life Technologies
S. aureus		
Mu50	VISA, MRSA, *mecA*$^{+}$Erm^{r}, ST5	NARSA
Mu50Δ*msaABCR*	*msaABCR* operon knock out	This study
Mu50 comp.	Mu50Δ*msaABCR* (pCN34-*msaABCR* operon)	This study
HIP6297	VISA, *mecA*$^{+}$, ST5	NARSA
HIP6297Δ*msaABCR*	*msaABCR* operon knock out	This study
HIP6297 comp.	HIP6297Δ*msaABCR* (pCN34-*msaABCR* operon)	This study
LIM2	VISA, *mecA*$^{+}$, ST247	BEI Resources
LIM2Δ*msaABCR*	*msaABCR* operon knock out	This study
LIM2 comp.	LIM2Δ*msaABCR* (pCN34-*msaABCR* operon)	This study
Plasmids		
pKOR1	Amp^{r}Cm^{r} ; Shuttle vector, temperature sensitive	Bae et al., 2006
pKOR1- Δ*msaABCR*	Upstream and downstream fragment of *msaABCR* operon cloned into pKOR1 for mutagenesis	This study
pCN34	Amp^{r} Erm^{r} ; Shuttle vector, Low copy number	Charpentier et al., 2004; NARSA
pHV1249	Cm^{r} ; 996-bp PCR fragment containing *cat194* gene cloned into pCN34 to generate pCN34(Cm^{r})	Petit et al., 1990
pCN34(Cm^{r})	Amp^{r}Cm^{r} ; used for trans - complementation of *msaABCR* operon	This study
pCN34(Cm^{r}) - *msaABCR* operon	1.7 kb PCR fragment containing *msaABCR* operon cloned into pCN34(Cm^{r})	This study

Table 3.2

Primers used in this study

Primers	Sequences (5' → 3')
Operon del 1	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCTTTAAATCAG CGATTAATGTTCGTTTG
Operon del 2	ATGACTGGATCCTATTAAAGACCCCTTCCATACTTCAAAAAC
Operon del 3	ATGACTGGATCCTTTCATGATGCTTGTTTAAAGTGTGGTAT
Operon del 4	GGGGACCACTTTGTACAAGAAAGCTGGGTAGTTTGGATTATC AATTCAATATGGCTTAGC
Comp-F1	GGGGGATCCTTTTACCACCTCATAATGTTAT
Comp-R1	CCCGAATTCAAATAAACAAAGTAATCCCCGA
Cm-F	GTTTAAGGGCCCACCTAGGTATTATCAAGATAAGAAAGAAA AG
Cm-R	CTATGACTCGAGGCCGCGGCCTTCTTCAACTAACGGGG
sigB-F	AAGTCCGGTACCACAGTATATAAAGAATCTGGTG
sigB-R	GGTTCCAGATCTAAATTCTATTTATGTGCTGC
RT-PCR Primers:	
gyrB RT F	CAACTATGAAACATTACAGCAGCGT
gyrB RT R	TGTGGCATATCCTGAGTTATATTGAAT
mprF RT F	GAACCACCGTTTTCAACTGAA
mprF RT R	GTAAATCTAACTCTGGCAACCATC
dltA RT F	ATGTTTAGCATCAGGCGGTAC
dltA RT R	ACTTGGGAAACGGCTCACTAA
rnaIII RT F	CTGAGTCCAAGGAAACTAACTCTAC
rnaIII RT R	TGATTTCAATGGCACAAGAT
graR RT F	GTTGCTGGTATTGAAGATTTCG
graR RT R	CGCCAAGTTCCATACTCATCAC
graS RT F	CACCTGTGACAGCCATGAAATTA
graS RT R	CATCAATGACCATGCGTTTAAGTGACA
walKR RT F	AAACAACTACAATCCCTTCATACTAA
walKR RT R	CTTGACGGTTGGCATACTCACTTAA
pbp4 RT F	CCGTTGGATTGACGAAATGT
pbp4 RT R	ACCAGCGATTTCGTTGATTT
gyrA RT F	CATTGCCAGATGTTCGTGAC
gyrA RT R	CCGGTGTCATACCTTGTTCA
sigB RT F	AACCGATACGCTCACCTGTC
sigB RT R	CGCGAACGAGAAATCATACA

Table 3.3

Vancomycin MIC of Wild types, msaABCR operon deletion mutants, and complemented mutants

Strains	Vancomycin MIC (μg/ml)			
	Mu50	HIP6297	LIM2	N315
Wild type	6.25	6.25	6.25	1.56
Δ*msaABCR*	1.56	1.56	1.56	1.56
Complemented mutant (pCN34-*msaABCR*)	3.13	3.13	3.13	1.56

Table 3.4

Cell wall thicknesses of wild types, msaABCR operon deletion mutants, and complemented mutants

Strains	Mean thickness of cell wall (nm ± SE)		
	Wild type	Δ*msaABCR*	Complemented mutant (pCN34-*msaABCR*)
Mu50	50.83 ± 3.82	24.23 ± .91	44.5 ± 3.98
HIP6297	41.8 ± 2.65	25.6 ± 1.61	51.4 ± 2.63

nm = nanometer; SE = Standard error of mean

CHAPTER IV

MOLECULAR AND PHENOTYPIC CHARACTERIZATION OF METHICILLIN-RESISTANT *Staphylococcus aureus* ISOLATES CAUSING BACTEREMIA AT A MAJOR HOSPITAL IN SOUTHERN MISSISSIPPI

Abstract

Staphylococcus aureus is the predominant cause of bacteremia worldwide. Distribution of methicillin-resistant *Staphylococcus aureus* (MRSA) tends to be geographically unique. We assessed molecular epidemiology and antibiotic resistance of MRSA isolates causing bacteremia in Southern Mississippi.

We collected 30 MRSA blood isolates over 1 year, which were subjected to staphylococcal chromosomal cassette *mec* (SCC*mec*) typing, pulsed field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and antibiotic resistance profiling. Risk factors underlying bacteremia were analyzed by Fisher's exact test and regression analysis.

All MRSA isolates were *mecA* positive and 70% were SCC*mec* type IV, indicating community-acquired infections, and 30% were SCC*mec* type II, of which 50% were Panton–Valentine leukocidin (PVL) negative, indicating hospital-acquired infections. Most isolates (97%) were resistant to oxacillin and erythromycin and 30% to clindamycin. Risk factor analysis revealed a strong association of prior hospitalization, prior antibiotic usage, nursing home origin, and presence of PVL with MRSA isolates with minimum inhibitory concentration (MIC) >1 μg/ml for vancomycin.

Diverse genetic backgrounds in terms of SCC*mec*, PFGE, and MLST types of MRSA were identified as causing bacteremia in Mississippi. A strong association of PVL with high vancomycin MIC is one of the important findings of our study.

Introduction

Staphylococcus aureus is a major human pathogen responsible for both community and hospital-associated infections. It is the causative agent of a wide array of diseases from skin and soft tissue infections (SSTIs) to more life-threatening conditions such as endocarditis, osteomyelitis, and septic arthritis. Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are causing increasingly complex public health problems in both community and hospital settings. Initially, MRSA strains were largely confined to hospitals and were referred to as hospital-acquired MRSA (HA-MRSA), causing treatment difficulties, especially in patients with prior exposure to antibiotics. During the past two decades, *S. aureus* infections have been worsened by the emergence of community-acquired MRSA (CA-MRSA) (94). The United States has an epidemic caused by CA-MRSA isolates (95-97). CA-MRSA isolates are clinically significant because they can also cause severe disease in individuals who are apparently healthy and without any predisposing risk factors (94, 97).

CA-MRSA isolates can be distinguished from HA-MRSA by molecular typing tools. Although all the MRSA isolates carry staphylococcal chromosomal cassette *mec* (SCC*mec*), the length of the SCC*mec* elements varies between CA- and HA-MRSA isolates. HA-MRSA isolates typically carry SCC*mec* types I, II or III, which are longer in size compared with types IV or V carried by CA-MRSA isolates. Additionally, HA-

MRSA isolates do not typically carry Panton–Valentine leukocidin (PVL) genes (*lukS-PV* and *lukF-PV*), whereas community-acquired isolates frequently carry PVL (94).

Bacteremia caused by MRSA isolates is associated with a higher risk of mortality compared with methicillin-susceptible *Staphylococcus aureus* (MSSA) (98). Given the importance of invasive MRSA infections in both community and healthcare settings, it is imperative to understand the clonal variation among clinical isolates. Isolates belonging to a certain clonal background have been shown to be more prevalent and virulent than others (99). In the United States, pulsed field type USA300 has been the dominant strain causing MRSA infections in community settings. Although studies by The Center for Disease Control and Prevention (CDC) indicated that USA100 used to be the predominant type causing clinical bacteremia, others reported that USA300 is becoming more dominant even in hospital settings (100). Tenover et al. (100) studied the prevalence of USA100 and USA300 isolates in nine geographical zones in the United States. The results showed that USA100 is predominant in mountain (60%), west north central (45%), east north central (50%) and New England (70%) regions. In contrast, USA300 was found to be predominant in the Pacific (49%), west south central (79%), South Atlantic (42%) and Mid-Atlantic (53%) regions. However, notably, there were no data reported from the Southeast United States (Mississippi, Alabama, Tennessee and Kentucky). As a result of this gap in the epidemiological data of MRSA isolates in this area, we carried out the first ever molecular and phenotypic characterization of MRSA isolates causing bacteremia in Southern Mississippi. A retrospective cohort study carried out between October 1995 and December 2003 showed that patients with MRSA bacteremia have more underlying diseases ($P = 0.02$), more severe sepsis in response to

their infection ($P < 0.01$), and consequently a higher mortality rate (101). Clearly, MRSA infection in blood indicates a more robust disease state. Therefore, it is important to study blood isolates to understand the degree of virulence of the MRSA isolates causing the epidemic.

The fact that relatively few antibiotics are available to treat MRSA infections has made the treatment more expensive and difficult. For many years vancomycin has been the drug of choice for MRSA infections. However, since the late 1990s, the situation has been exacerbated by the emergence of vancomycin intermediate-resistant and resistant *S. aureus* strains. Continued use of vancomycin has been shown to accelerate the appearance of resistant sub-clones both in the laboratory (77) and in hospital settings (29, 36, 75). Hence, for effective treatment with vancomycin, early detection of underlying risk factors that may be associated with the increased vancomycin resistance is important. In this study, we isolated and collected 30 bacteremia-causing MRSA isolates, analyzed their clonality by molecular methods, and assessed their antibiotic resistance profile. We also analyzed the association of predisposing risk factors with the outcome of increased vancomycin resistance in certain strains.

Materials and Methods

Research ethics

The study was approved by the Institutional Review Board of the University of Southern Mississippi and the Forrest General Hospital. Based on the protocol used, a waiver of informed consent was granted.

Collection and maintenance of isolates

Clinical isolates of MRSA strains from patients were collected from the microbiology laboratory of a large referral level II trauma hospital in Forrest County in Southern Mississippi. Isolates were collected between March 2013 and February 2014. Once an MRSA organism was isolated from a positive blood culture, two or three colonies were suspended into the Prompt Inoculation System-D (Siemens Medical Solutions, Malvern, PA, USA). This is a microdilution procedure for susceptibility testing that helps to determine the standardized inoculum needed for MicroScan. The suspension was inoculated onto the MicroScan Gram Positive panel (Siemens Medical Solutions) and loaded onto the MicroScan instrument for incubation, followed by addition of reagents, and reading of the panel. The following day, the panel was processed and observed by an experienced technologist to confirm the result and purity of the culture. Once the isolates were confirmed as MRSA, they were re-streaked and maintained in DMSO stocks at –80°C. A database of information about the source of infections was maintained using de-identifiers.

Identification of virulence factors: mecA, PVL and hlg

The presence of *mecA*, PVL and *hlg* in the MRSA isolates was tested using polymerase chain reaction (PCR). Chromosomal DNA of the isolates was amplified with the primers listed in Table 4.1. The presence of a band in agarose gel from the respective PCR compared with a positive control indicates the presence of the gene.

Pulsed field gel electrophoresis (PFGE)

PFGE was performed as described previously (102). *Sma*I-digested chromosomal DNA of the bacterial isolates was run on PFGE. Gel images were analyzed and a

dendrogram was generated in Bionumerics (version 5.0.1, Applied Maths, Austin, TX, USA) using 80% similarity cutoff. Banding patterns were compared with standard banding patterns of known PFGE types of *S. aureus* determined by McDougal et al. (102).

Multilocus sequence typing (MLST)

MLST of the MRSA isolates was performed as described previously (103). Seven housekeeping genes were amplified and sequenced using the primers listed in Table 4.1. Sequence data for each gene were individually analyzed to determine the sequence type for isolates according to the criteria provided for *S. aureus* using the web-based tool MLST (http://www.mlst.net).

SCCmec typing

SCC*mec* typing was performed as described by Ghaznavi-Rad et al. (104). A multiplex PCR was performed using the chromosomal DNA of the isolates as a template and a primer mastermix containing all the primers at a final concentration of 100 nM, using a Qiagen Multiplex PCR kit (Qiagen, Valencia, CA, USA). The primers used for SCC*mec* typing are listed in Table 4.1. The PCR product was run in 2.0% agarose gel. SCC*mec* type of the isolates was determined by comparing the band pattern of the PCR product with the standard pattern as described previously (104).

Antibiotic susceptibility testing

Antibiotic susceptibility of all isolates was tested in triplicate using the broth microdilution method according to the Clinical and Laboratory Standards Institute guidelines (64). Mueller–Hinton broth supplemented with 2% NaCl was used for all broth microdilution experiments. Antibiotic-containing wells were inoculated with 5 ×

10^5 CFU/ml bacteria (65). After overnight incubation at 35°C, wells were analyzed for visible bacterial growth as exhibited by turbidity. The lowest concentration of antibiotic that prevented bacterial growth was considered to be the minimum inhibitory concentration (MIC). The antibiotics used in this study were vancomycin, oxacillin, erythromycin, clindamycin, rifampin, amoxicillin/clavulanic acid (Augmentin), trimethoprim/sulfamethoxazole (Bactrim), and linezolid. Isolates were considered resistant or sensitive based on the antibiotic breakpoints given on DAILYMED (dailymed.nlm.nih.gov).

Risk factor analysis

The medical records of all selected blood cultures positive for MRSA were reviewed for the following variables: demographics (age, sex, race, and body mass index); comorbidity (diabetes mellitus, chronic skin condition, chronic kidney disease, chronic heart failure, obesity, immunosuppression, and HIV infection); invasive procedures (surgery, dialysis, intravenous catheterization, and mechanical ventilation); history (smoking, drug usage, prior hospitalization, and antibiotic usage); and source of bacteremia.

Data analysis and statistical tests

For analysis of the collected data, SCC*mec*, PFGE and MLST types were combined for each isolate. From the antimicrobial resistance data, the resistance pattern of each isolate was determined. The combined molecular types were then cross-tabulated with antimicrobial resistance patterns. Associations between molecular types and resistance pattern, between MLST and PFGE types, and between SCC*mec* and PFGE types were determined by Fisher's exact test. A P value < 0.05 was considered

significant. All statistical analyses were performed using SPSS version 20. Risk factors were binary coded in SPSS. To measure the association between risk factors and vancomycin MIC >1 μg/ml, a crude analysis was first performed by Fisher's exact test. This test was chosen over χ^2 because of the small sample size. The factors that displayed a *P* value < 0.2 were used to perform binary logistic regression to determine the odds ratio (OR).

Results

Overview

During the period between March 2013 and February 2014, a total of 322 clinical MRSA isolates were collected from a regional hospital in Southern Mississippi. Thirty blood isolates were selected for this study. Among all the blood cultures in the hospital, the incidence of MRSA infection was estimated to be 49 for every 10 000 patients. The proportion of blood MRSA isolates was estimated to be 7.1% of all MRSA isolates.

Clinical and epidemiological data

The majority of patients (72%) had a history of previous antibiotic usage. Regarding the source of infection, pneumonia was found to be the most common source of bacteremia (28.5%), followed by osteomyelitis (17.8%), and dialysis catheter infection (10.7%). Diabetes mellitus was found to be the most common comorbidity among the patients (51.7%). Details of the demographic data are listed in Table 4.2.

Pulsed field types and multilocus sequence types

PFGE has been a valuable tool for investigating *S. aureus* outbreaks, especially in hospitals because of its high resolving power (105-111). McDougal et al. (102) described USA typing as a nomenclature system for PFGE typing of *S. aureus* isolates. They

identified eight distinct lineages (USA100–800) that incorporate both oxacillin-resistant and -sensitive strains. Since then, USA types have been used to describe the epidemiological spread of pathogenic *S. aureus* strains worldwide.

In the present study, using an 80% similarity cutoff, blood isolates of MRSA belonged to two major clusters designated A and B (Figure 4.1). Cluster A was composed of isolates with USA100 and USA800 types, whereas Cluster B was composed of USA300 and USA700 type isolates. One isolate (USMFG160) was novel; however, it appeared closest to USA800 isolates in the PFGE typing. Overall, USA300 was found to be the most common pulsed field type (40%) among the bacteremia-causing MRSA isolates. USA100, 700 and 800 accounted for 23.3, 20 and 13.3% of the isolates, respectively (Figure 4.2 A).

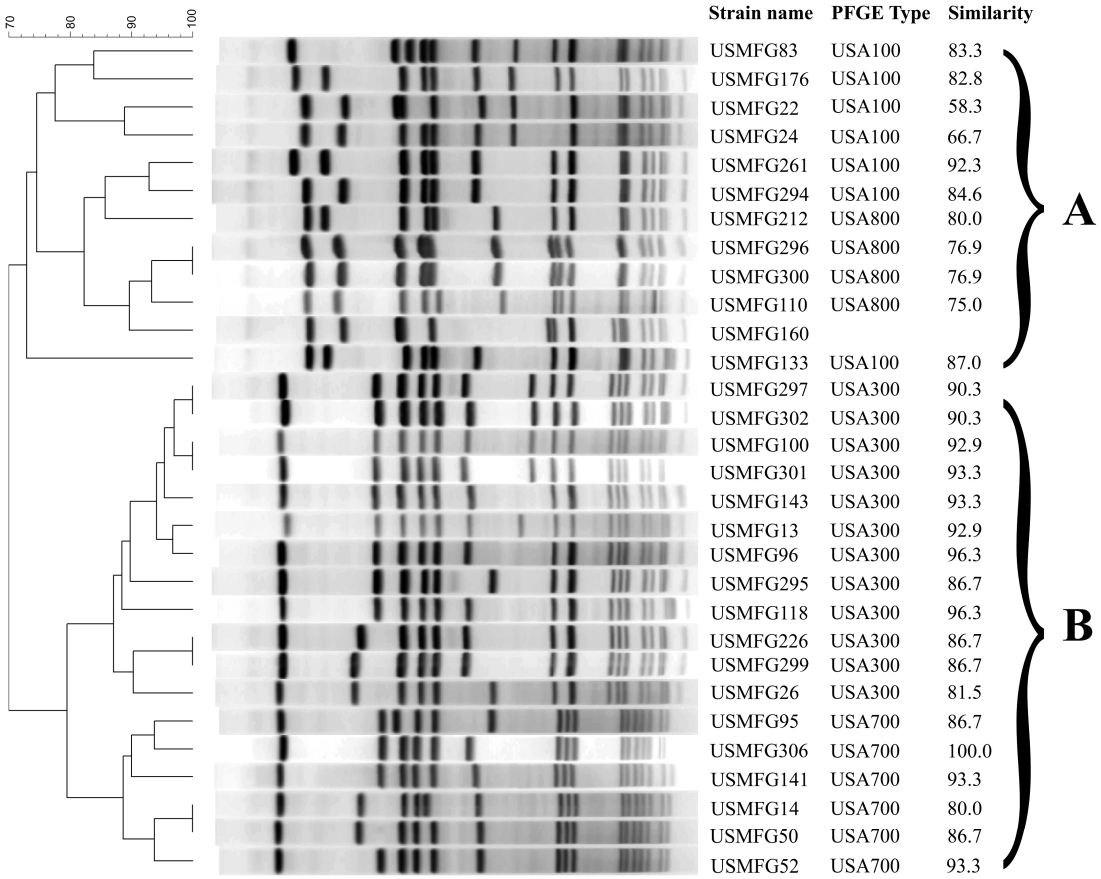

Figure 4.1. PFGE pattern and phylogenetic tree of the MRSA isolates

MLST is based on sequence analysis of seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi* and *yqiL*) of *S. aureus*. It gives an indication of clonal evolution of MRSA. Different sequences of these genes are assigned unique allele numbers. Combination of all seven genes resulted in an allelic profile or sequence type. In the present study, blood isolates of MRSA belonged to five distinct sequence types. Among these, ST5 accounted for 40% of the isolates, constituting the largest group. ST8, ST72 and ST100 were found in 37, 20 and 3% of the isolates, respectively (Figure 4.2 B). One isolate (USMFG95) was novel. McDougal et al. (102) have observed a strong association between the PFGE and MLST types of MRSA isolates. They found that each pulsed field

type could be assigned a unique MLST type such as USA100 and USA800 sharing ST5, and USA300 and USA700 isolates were ST8 and ST72, respectively. Our results largely reflected these findings. For example, all USA100 and USA800 isolates were found to be ST5. All but one USA300 isolates were found to be ST8, and only one (USMFG100) was determined to be ST100. All but one USA700 isolates were found to be ST72. There was a strong association between MLST and PFGE types ($P < 0.001$) of the blood isolates of MRSA.

SCCmec types of bacteremia isolates

The methicillin resistance of *S. aureus* is caused by the presence of the *mecA* gene that encodes a 78-kDa penicillin-binding protein (PBP2a or PBP2′). Compared to other PBPs, PBP2a has a lower affinity for β-lactam antibiotics. As a result, even in the presence of these antibiotics, the peptidoglycan layer is not disrupted and the bacterium survives (11, 12). The *mecA* gene is housed within the *mec* operon together with its regulatory genes *mecI* and *mecR1* (11). Since the first detection of the SCC*mec* element from N315 in 1999, multiple *mec* types have been identified because of the hypervariability of SCC*mec* elements. Nine SCC*mec* types (I–VIII and V_T) have been detected to date. These are distinguished by the *ccr* gene complex, which helps in excision and insertion of SCC*mec* elements (13-15). Clones of unique SCC*mec* types have driven the epidemic of MRSA worldwide. For instance, SCC*mec* type I was first discovered in the United Kingdom in 1961 and spread around the world during the 1960s. Following that in 1982, SCC*mec* type II was discovered in Japan and this clone also spread worldwide. During the 1990s SCC*mec* type IV caused a global epidemic.

Therefore, in addition to MLST and PFGE, SCC*mec* typing can reveal information pertinent for epidemiological analysis of MRSA strains (12).

In our analysis, all blood isolates were found to possess the *mecA* gene (Table 4.3), and 67% of the isolates were found to carry SCC*mec* type IV by multiplex PCR (Figure 4.2 C). All but one USA300 strains were *mec* type IV. This isolate was found to be *mec* type II. All the USA700 and USA800 isolates also possessed *mec* type IV. USA100 isolates were found exclusively to possess *mec* type II, which accounted for 29% of the blood isolates. Only one isolate, USMFG160, which was identified as novel by PFGE, was found to possess *mec* type V (3%). A strong association was found between the PFGE and SCC*mec* types of the blood isolates of MRSA ($P < 0.001$).

Frequency of PVL and hlg in bacteremia isolates

PVL is capable of lysing the cell membrane of human neutrophils. Although its role in pathogenesis is controversial, PVL has been strongly associated with several CA-MRSA infections (112-115). Naimi et al. (10) showed with a large number of MRSA cases that while 77% of CA-MRSA possessed the PVL gene, only 6% of HA-MRSA were positive for PVL. Despite its strong correlation with CA-MRSA, it is not clear whether PVL contribute to the fitness or virulence of the CA-MRSA strains (94). HA-MRSA strains are typically characterized by SCC*mec* type II and an absence of PVL. Therefore, despite the controversy about its function, the presence or absence of PVL genes may be important to determine if a particular isolate is community or hospital acquired. Hemolysin gamma (γ) (*hlg*) gene is another virulence factor, which has a sequence similarity with PVL genes and functions as two component cytotoxins.

In our study, all USA300 strains were PVL positive (Table 4.3). USA100 strains (SCC*mec* type II) were mostly found to be PVL negative, except for two isolates (USMFG23 and 24). It is likely that USA100, *SCCmec* II, and PVL-negative strains are HA-MRSA (17% of all isolates). About 50% of all USA700 and USA800 isolates were found to be PVL positive, while the rest were PVL negative. All isolates were found to be *hlg* positive (Table 4.3).

Antibiotic susceptibility analysis

All isolates in this study were found to be resistant to oxacillin and amoxicillin/clavulanic acid. The majority of the isolates (93%) were resistant to erythromycin followed by 30% resistant to clindamycin. All isolates were found to be sensitive to vancomycin, rifampin, trimethoprim/sulfamethoxazole, and linezolid. Although we did not find any vancomycin intermediate or resistant strains, we noted that some isolates had higher MIC for vancomycin than others. Specifically, while 13 isolates had MIC of 0.5 μg/mL, seven had MIC of 1 μg/mL, and 10 had MIC >1 μg/ml for vancomycin (Table 4.4). Our antibiotic susceptibility analysis of blood isolates reflected the susceptibility record maintained by the hospital in the period between January and June 2013, which revealed that all MRSA isolates tested were resistant to oxacillin and amoxicillin/clavulanic acid. Ninety-one percent were resistant to erythromycin, 30% to clindamycin, and 1% to trimethoprim/sulfamethoxazole. All MRSA isolates were sensitive to vancomycin and linezolid. There was no significant association between antibiotic resistance patterns and combined molecular types (PFGE/MLST/SCC*mec*) of strains ($P = 0.140$). In other words, antibiotic resistance patterns were distributed across all different molecular types (Figure 4.2 D).

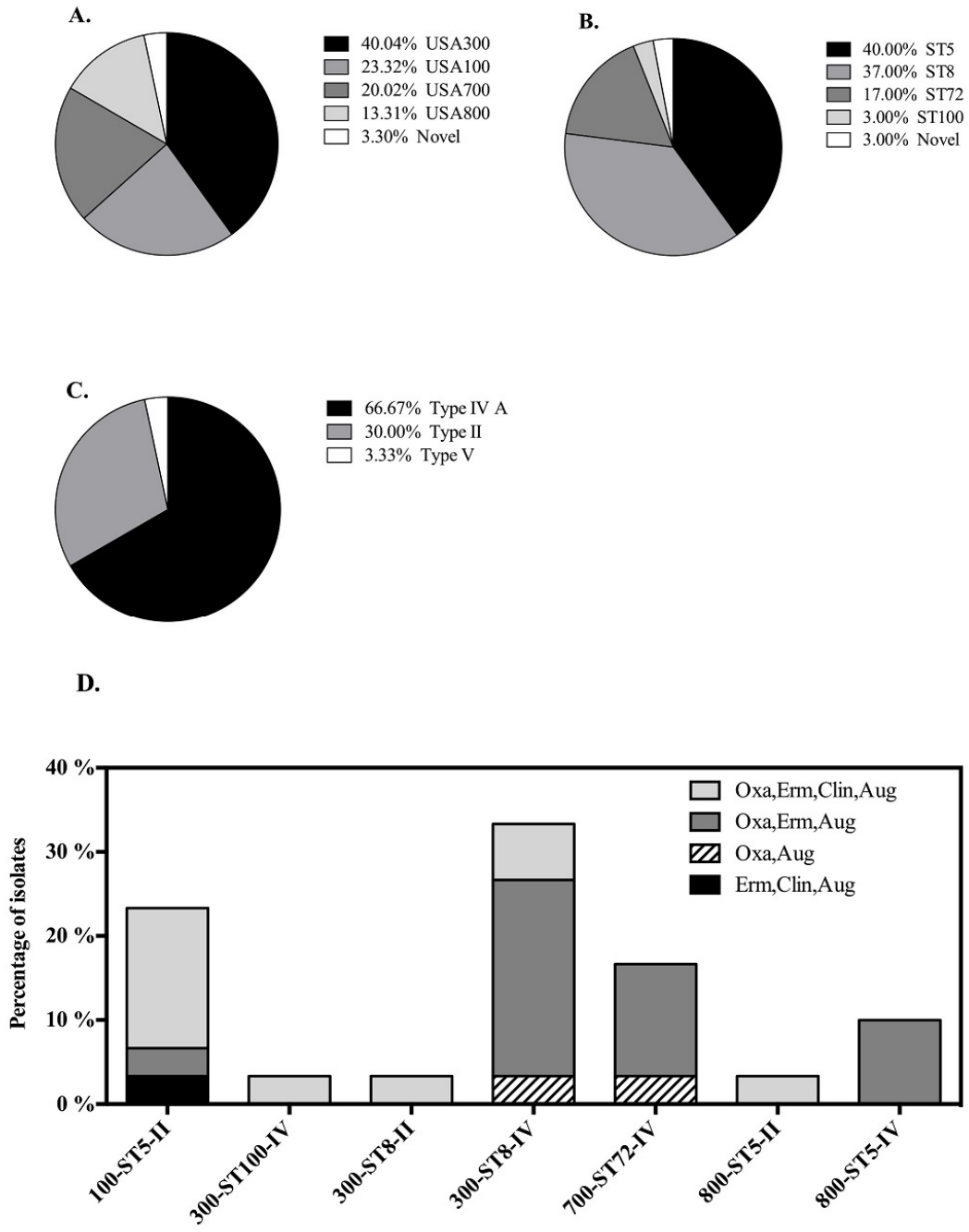

Figure 4.2. A. Distribution of PFGE types among the isolates causing bacteremia in Southern Mississippi; B. Distribution of MLST types among bacteremia-causing isolates; C. Distribution of SCC*mec* types in bacteremia-causing MRSA isolates; D. Distribution of resistance patterns among the different clonal variations of the bacteremia-causing MRSA isolates.

Risk factors

Vancomycin is the most frequently used drug in treating bacteremia caused by MRSA. Most of the patients who developed bacteremia had a history of antibiotic usage (Table 4.5). Moreover, as mentioned earlier, we found 10 isolates (33%) with MIC >1 μg/ml for vancomycin from our susceptibility analysis (Table 4.4). Among these, five isolates showed MIC of 2 μg/ml. Repeated use of vancomycin gives rise to resistant sub-clones with increased MIC compared with that of susceptible strains, and results in more complicated, life-threatening conditions (77). We aimed to identify any predisposing risk factors associated with this phenomenon. A detailed review of the medical records of the patients and statistical univariate analysis (crude analysis) revealed that several risk factors were associated with MIC >1 μg/ml for vancomycin. Table 4.5 lists all the risk factors analyzed in this study. The factors with $P < 0.2$ in the univariate analysis were further analyzed by logistic regression to determine OR. The presence of the PVL gene was strongly associated with vancomycin MIC >1 μg/mL ($P = 0.01$) and showed a high OR in regression analysis. With the exception of smoking, all the other factors revealed an OR >1. This suggests that the odds of having an MRSA isolate with vancomycin MIC >1 μg/ml will increase in patients with prior usage of antibiotics, prior hospitalization, coming from a nursing home, and being infected by a strain possessing PVL.

Discussion

To the best of our knowledge, this is the first study to assess systematically the clinical and molecular epidemiology of bacteremia by MRSA isolates in Mississippi. The hospital where the isolates were collected is a referral healthcare facility in Southern Mississippi. Therefore, the patients are from a broad geographical area. Few studies of

MRSA have been performed in Mississippi. A CDC study at a prison in Mississippi revealed MRSA infections among 4.9% of the inmates. Among 59 cases tested, 41 were confirmed MRSA by CDC (116). However, limited molecular analysis was done.

Our analysis showed that the majority of bacteremia cases were caused by USA300-ST8-SCC*mec* IV isolates. This predominance of USA300 was observed in studies done in other regions of the United States as well. Tenover et al. (100) analyzed 493 isolates from 23 US laboratories by PFGE typing. They found that USA300 was predominant in the Pacific, west south central, South Atlantic and mid-Atlantic regions, whereas in the mountain, west north central and east north central regions, USA100 was predominant. However, the authors noted that the percentage of USA300 in the blood isolates increased significantly compared with a previous study performed by CDC-Active Bacterial Core surveillance (ABCs) in 2005–2006 with 1984 blood isolates. The predominance of CA-MRSA over HA-MRSA in the hospital setting has been mathematically modeled by D'Agata et al. (117). They predicted that CA-MRSA would eventually become dominant in hospitals. An increasing community reservoir of CA-MRSA strains results in a greater influx of CA-MRSA-infected patients into hospitals, which contributes greatly to the dominance of CA-MRSA over HA-MRSA. Moreover, CA-MRSA isolates carry smaller SCC*mec* genes, do not carry antibiotic resistance genes unlike HA-MRSA strains, and grow more rapidly than HA-MRSA, which combined gives CA-MRSA strains a competitive advantage and increased chance of colonizing, to eventually outcompete HA-MRSA. Our observation from the hospitalized patients confirms the predominance of USA300 isolates (CA-MRSA) in bacteremia-causing isolates in our region.

Although USA300 comprised the major portion of the isolates, regarding antibiotic resistance, USA100-ST5-SCC*mec* II isolates were more resistant against multiple drugs. Many of these isolates were PVL negative. USA300 isolates are typically susceptible to fluoroquinolones and clindamycin (102). However, in this study, we found that 30% of USA300 isolates were clindamycin resistant. This supports previous observations in other studies that reported emerging resistance of USA300 isolates against clindamycin (95, 118-120).

An important finding of our study was the strong association of the PVL genes (*lukS-PV* and *lukF*-PV) with elevated MIC for vancomycin. In the univariate analysis of association of risk factors, we found that prior antibiotic use ($P = 0.03$) and presence of PVL ($P = 0.01$) had a strong association with vancomycin MIC >1 μg/ml. A high OR (>1) in the regression analysis indicates that prior antibiotic use by a patient or presence of PVL genes in an isolate increases the chance of vancomycin MIC >1 μg/ml. Prior antibiotic use is known to be a leading cause of the emergence of vancomycin resistance in a hospital setting (29, 36, 75). However, the contribution of PVL genes in the resistant isolates is unclear. PVL is a leukocidin that is capable of killing human neutrophils by lysing their cell membranes. Although it is regarded as a virulence factor for CA-MRSA isolates, contradictory findings have been reported (112-115, 121). Voyich et al. (112) and Wardenburg et al. (113) showed that the presence or absence of PVL did not affect the capacity of a strain for sepsis formation or mortality rate in a mouse model. In contrast, Labandeira-Ray et al. (114) showed overexpression of PVL caused necrotizing pneumonia in a mouse model, suggesting that PVL contributes significantly to the infection process of MRSA. Diep et al. (115) showed that PVL containing wild-type

strains might possess a survival benefit in the early hours of infection over the PVL-negative mutants. Hongo et al. (121) showed that although PVL did not lyse mouse neutrophils, anti-PVL antibodies were able to reduce the lysing activity of USA300 and USA400 strains against human neutrophils, suggesting that PVL may be important for CA-MRSA infection in humans, and that mice may not be suitable model for this study. David et al. (94) pointed towards a variation in the amino acid sequence of PVL genes in the MRSA strains to be the possible reason for these contradictory results. However, any relation between the presence of PVL genes and vancomycin MIC has not been studied, and further research is needed. Although it is unclear why PVL is strongly associated with elevated vancomycin MIC, it may have been due to our relatively small sample size of MRSA-infected patients, which may have resulted in skewed observation. Another limitation of this study was that the isolates were collected from a group of ill individuals, and less severely infected cases may have other risk factors that were not discovered in this study.

In conclusion, to the best of our knowledge, this is the first report of systematic molecular epidemiological analysis of MRSA isolates in Mississippi. Collectively our data show the presence of both CA-MRSA and HA-MRSA isolates among bacteremia-causing MRSA in this region. The prevalence of USA300 isolates in a hospital setting is an extension of a nationwide observation and mathematical prediction; however, the association of PVL genes as a risk for vancomycin MIC warrants more detailed investigation.

Table 4.1

Primers used in this study

Experiment	Primer name	Sequence (5' to 3')	Reference
SCC*mec* typing	Type I F	GCTTTAAAGAGTGTCGTTACAGG	Ghaznavi-Rad et al.(104)
	Type I R	GTTCTCTCATAGTATGACGTCC	
	Type II F	GATTACTTCAGAACCAGGTCAT	
	Type II R	TAAACTGTGTCACACGATCCAT	
	Type III F	CATTTGTGAAACACAGTACG	
	Type III R	GTTATTGAGACTCCTAAAGC	
	Type IVa F	GCCTTATTCGAAGAAACCG	
	Type IVa R	CTACTCTTCTGAAAAGCGTCG	
	Type IVb F	AGTACATTTTATCTTTGCGTA	
	Type IVb R	AGTCATCTTCAATATGGAGAAAGTA	
	Type IVc F	TCTATTCAATCGTTCTCGTATT	
	Type IVc R	TCGTTGTCATTTAATTCTGAACT	
	Type IVd F	AATTCACCCGTACCTGAGAA	
	Type IVd R	AGAATGTGGTTATAAGATAGCTA	
	Type IVh F	TTCCTCGTTTTTTCTGAACG	
	Type IVh R	CAAACACTGATATTGTGTCG	
	Type V F	GAACATTGTTACTTAAATGAGCG	
	Type V R	TGAAAGTTGTACCCTTGACACC	
	SA442 F	AATCTTTGTCGGTACACGATATTCTTCACG	
	SA442 R	CGTAATGAGATTTCAGTAGATAATACAACA	

Table 4.1 (continued).

Experiment	Primer name	Sequence (5' to 3')	Reference
Multilocus sequence typing	*arcC*-Up	TTGATTCACCAGCGCGTATTGTC	Enright et al.(103)
	arcC-Dn	AGGTATCTGCTTCAATCAGCG	
	aroE-Up	ATCGGAAATCCTATTTCACATTC	
	aroE-Dn	GGTGTTGTATTAATAACGATAT	
	glpF-Up	CTAGGAACTGCAATCTTAATCC	
	glpF-Dn	TGGTAAAATCGCATGTCCAATTC	
	gmk-Up	ATCGTTTTATCAGGACCATC	
	gmk-Dn	TCATTAACTACAACGTAATCGTA	
	pta-Up	GTTAAAATCGTATTACCTGAAGG	
	pta-Dn	GACCCTTTTGTTGAAAAGCTTAA	
	tpi-Up	TCGTTCATTCTGAACGTCGTGAA	
	tpi-Dn	TTTGCACCTTCTAACAATTGTAC	
	yqiL-Up	CAGCATACAGGACACCTATTGGC	
	yqiL-Dn	CGTTGAGGAATCGATACTGGAAC	
PCR of PVL	*luk-PV*-F	ATCATTAGGTAAAATGTCTGGACATGATCCA	
	luk-PV-R	GCATCAACTGTATTGGATAGCAAAAGC	
PCR of *hlg*	*hlg*-F	GCCAATCCGTTATTAGAAAATGC	
	hlg-R	CCATAGACGTAGCAACGGAT	
PCR of *mecA*	*mecA*-F	AAAATCGATGGTAAAGGTTGGC	
	mecA-R	AGTTCTGCAGTACCGGATTTGC	

Table 4.2

Demographic characteristics of patients with MRSA bacteremia

Characteristics	*N* (valid %)
Gender	
Male	16 (55.2)
Female	14 (44.8)
Age ≥ 65 years	11 (39.3)
Race	
Caucasian	14 (48.3)
African American	15 (53.5)
Other	0 (0)
Service in First 48 hours	
Intensive care unit	11 (37.9)
Other	18 (62.1)
Comorbidities	
Diabetes mellitus	15 (51.7)
Chronic skin condition	4 (13.8)
Chronic kidney disease	12 (41.4)
Chronic heart failure	8 (26.7)
Obesity	11 (36.7)
BMI ≥ 30	11 (36.7)
Immunosuppression	2 (6.9)
HIV	3 (12)
Invasive procedures	
Surgery	3 (10.3)
Dialysis	6 (20.7)
Intravenous catheter	8 (26.7)
Mechanical ventilation	3 (10.3)
History	
Smoking	11 (37.9)
Injection drugs	1 (3.4)
Hospitalization	12 (41.4)
Prior antibiotic usage	21 (72.4)
Long term care facility	6 (20.7)
Source of bacteremia	
Pneumonia	10 (35.7)
Osteomyelitis	5 (17.8)
Dialysis catheter and fistula related infection	4 (14.3)
Cellulitis	4 (14.3)
Central line infection	3 (10.7)
Septic arthritis	1 (3.6)
Uterine tract infection	1 (3.6)

Table 4.3

Percentage of PVL, mecA and hlg virulence factors of isolates belonging to each combination type (PFGE-MLST-SCCmec)

Combination types	Presence or absence of virulence factors					
	PVL		*mecA*		*Hlg*	
	+	−	+	−	+	−
100-ST5-II	7%	17%	23%	-	23%	-
300-ST8-II	3%	-	3%	-	3%	-
300-ST8-IV	33%	-	33%	-	33%	-
300-ST100-IV	3%	-	3%	-	3%	-
700-ST72-IV	10%	7%	17%	-	17%	-
700-Novel-IV	-	3%	3%	-	3%	-
800-ST5-II	3%	-	3%	-	3%	-
800-ST5-IV	3%	7%	10%	-	10%	-
Novel-ST5-V	3%	-	3%	-	3%	-

Table 4.4

Number of isolates possessing different MIC for vancomycin

Vancomycin MIC (µg/ml)	Number of isolates (%)
0.5	13 (43.3%)
1	7 (23.3%)
1.5	5 (16.6%)
2	5 (16.6%)

Table 4.5

Univariate (crude) analysis of the association between risk factors and MRSA isolates with MIC >1 μg/mL for vancomycin

Variables	Fishers Exact test *p* value
Age	1
Sex	0.45
Weight	1
Service in first 48 hours	1
Diabetes mellitus	0.42
Chronic kidney disease	0.23
Chronic heart failure	0.38
Obesity	0.69
BMI	0.69
Immunosuppression	1
Mechanical ventilation	1
HIV	1
Use of injection drug	0.31
Smoking	0.09
Prior antibiotic use	0.03**
Prior Hospitalization	0.1*
Prior surgery	1
Long term care facility	0.33
Dialysis	0.33
Intra-venous catheter	0.2
Nursing home	0.07*
Positive MRSA culture	0.2
Positive nasal swab	0.28
Presence of PVL	0.01**
Presence of HLG	0.33
SCC*mec* type	0.47
Clonal type	0.38
MLST type	0.27

* *P* values less than 0.2; ** *P* values less than 0.05

CHAPTER V

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF MsaB PROTEIN

Introduction

The *msaABCR* operon has been described to be consisting of four genes namely *msaA*, *msaB*, *msaC,* and an antisense RNA *msaR*. Of these, *msaA* and *msaC* were found to be non-coding RNA and only effector protein was found to be encoded by *msaB* (56). The MsaB protein is believed to execute the regulatory function of the *msaABCR* operon through its interaction with global regulators such as *sarA*, *agr*, *sigB,* and other genes. MsaB protein (previously called CspA) is predicted to contain a cold shock DNA binding domain (CSD) and bears homology to *E. coli* and *B. subtilis* cold shock proteins (CSPs). Bacteria respond to abrupt decrease in temperature by changing the protein expression patterns (21). Although expression of most cellular proteins decrease during the cold stress, expression of cold shock proteins reaches its peak at this time. One of the first proteins described to express under cold shock is the *E. coli* cold shock protein CspA (22). Many more proteins have been discover afterwards, possessing homology to the *E.coli* CspA protein. This includes *Bacillus subtilis* proteins CspB, CspC, and CspD (122). Sequence alignment of CSPs from several different organisms revealed that they possess a significant sequence homology with eukaryotic gene regulatory Y-box factors. Y-box proteins are DNA binding proteins are known to regulate transcription and translation (123). Although most CSPs are found to be expressed during cold shock, CspE and CspC are found to express constitutively at normal growth conditions at 37°C. Moreover, the constitutively expressed CSPs were thought to be regulating two major stress response proteins of *E. coli* RpoS and UspA (23). *Staphylococcus aureus* possesses

three CSP family proteins. All of them bears significant homology to the *E. coli* CspA. Katzif et al in their studies have suggested that the *S. aureus* CspA (or MsaB) is involved in the regulation of pigment production and susceptibility to antimicrobial peptide of human cathepsin-G (124, 125). The ability of *S. aureus* to cause a wide array of infections is due to its ability to evade or defend itself from host immune system (126). During Invasion, *S. aureus* has the ability to sustain the response triggered by the host, including the harmful reactive oxygen species (ROS) produced by neutrophils and macrophages, which are key cells of the human innate immune system. A study by Liu et al suggested that the golden pigment produced by *S. aureus,* called staphyloxanthin plays a significant role in susceptibility of *S. aureus* to reactive oxygen species (ROS) and neutrophil killing (127). In that study, the Δ*crtMN* mutants that is deficient in pigment production was found to be more susceptible to killing by ROS and by neutrophil phagocytosis. Therefore MsaB could be indirectly or directly linked to the immune evasion processes during stressed conditions. Moreover, MsaB has also been found to be playing significant role in biofilm formation, antibiotic resistance, small colony variance formation, and capsule formation. These are thought to be the basis for *S. aureus* long term survival and persistence inside the host. Sahukhal et al suggested that the Δ*msaABCR* operon mutant of the community–acquired MRSA strain USA300 is defective in biofilm formation (128). In an unpublished study, I observed that whereas wild type *S. aureus* strain Mu50 is able to produce small colony variants, the *msaABCR* mutant of it is unable to produce small colonies. These observations indicate that *msaABCR* mutant may be defective in the stress response regulation. As mentioned before, MsaB is found to be the only protein produced from the *msaABCR* operon, this

suggests that MsaB may be responsible for stress response regulation in *S. aureus*. In this study, we expressed and purified MsaB protein and characterized the structure and its role in responding to stress conditions.

Materials and Methods

Cloning, expression, and purification of the MsaB protein

The *msaB* open reading frame of *S.aureus* strain USA300-LAC was cloned into pH6HTNHis$_6$HaloTag® T$_7$ plasmid (Promega Corporation, Madison, WI) as an *XbaI-ApaI* fragment. The resulting plasmid pH6HTN-*msaB* was then transformed into *E. coli* BL21-DE3 strain and transformants were selected in LB-agar plate with ampicillin. 10 ml overnight culture of a positive colony was then inoculated into 1 Liter LB broth with 100 µg/ml ampicillin.

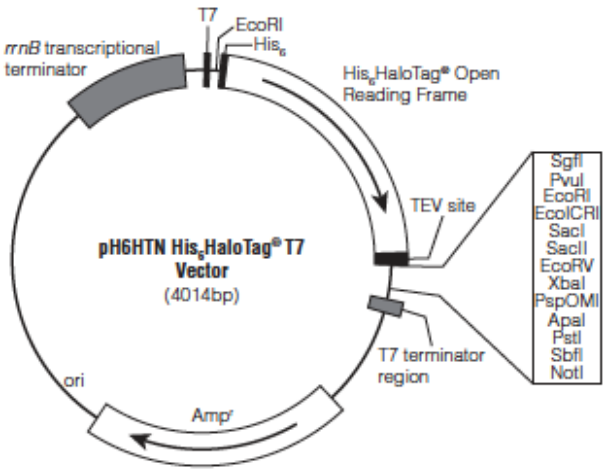

Figure 5.1. Plasmid map of pH6HTN His$_6$HaloTag

When the cells reach OD_{600} = 0.4, Protein expression was induced by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM. 5 hours post induction, the cells were pelleted, resuspended in PBS, pH 7.4, supplemented

with protease inhibitor cocktail (Roche), and lysed by sonication. Cell lysate was then centrifuged at 10,000 ×g for 30 minutes to remove cell debris.

The His_6Halo-MsaB fusion protein was purified from the clear lysate using a nickel column (HisPur^{TM} Ni-NTA resin, Thermo Scientific, Rockford, IL). The fusion protein was eluted from the column with phosphate buffered saline supplemented with 250 mM imidazole. Imidazole was removed from the protein sample by dialyzing it against pure PBS for overnight. The fusion protein was then cleaved using tobacco etch virus (TEV) protease (AcTEV Plus, Life Technologies) to remove the His_6Halo tag. 1 unit of the enzyme was used for 10 μg of protein. 20% glycerol was added to the cleavage reaction to stabilize the protein. The cleavage reaction was carried out in 4°C for overnight.

Finally, MsaB protein was purified from the tag using reversed phase HPLC. A C-18 column was used for HPLC. The column was first activated by washing with 100% acetonitrile (Acn) supplemented with 0.01% trifluoroacetotic acid (TFA). After equilibrating the column with 100% H_2O supplemented with 0.01% TFA, sample (1 ml) was applied to the column at a rate of 1 ml/minute. The column was then washed/eluted with a gradient of acetonitrile as follows: 0 – 2% Acn in 2 mins; 2 – 30% in next 2 mins; 30 – 70% Acn in next 12 mins; 70 – 100% Acn for next 2 mins, and finally, 100% acn for 2 mins. 1 ml fractions were collected throughout the process. Fractions that showed sharp peaks at 280 nm were collected and analyzed by polyacrylamide gel electrophoresis. MsaB fraction was determined by molecular weight and mass–spectrometry. After removal of acetonitrile from the correct fraction by vacuum centrifuging, the protein was dissolved in Tris-HCl buffer pH 7.4 and stored in -80°C for future use.

Antibody against MsaB was raised in rabbit using a commercial source (Pierce Biotechnologies, USA). A 70 – Day bleed of the animals immunized with MsaB was purified by column chromatography to isolate pure anti–MsaB antibody.

Circular Dichroism of MsaB

Far-UV CD spectra of MsaB were collected on a Jasco J-815 CD spectro-polarimeter using a 0.1 mm path length quartz cuvette (Precision cell). The samples were monitored in a continuous scan mode from 260 to 198 nm with a scanning speed of 50 nm/min, with a data integration time of 8 s and with 1 nm bandwidth, while the data pitch was maintained at 0.1 nm. For each data set, 12 scans were averaged and were subjected to buffer subtraction. The buffer-corrected scans were then smoothened using the Savitzky-Golay algorithm with the convolution width of 25 using the Jasco spectrum analysis program.

Matrix–Assisted Laser Desorption/Ionization — Time of Flight (MALDI-ToF) analysis of MsaB

The MsaB protein sample was analyzed using MALDI-ToF mass spectrometer (Bruker Daltonics Inc). The sample containing 40 picomoles of MsaB (1 µL) was mixed with 1 µL of matrix made of 10 mg sinnapinic acid (SA) dissolved in 1:1 acetonitrile: water and 0.1% TFA. A 1 µL aliquot of this mixture was then spotted in triplicates on to a MSP 96 microchip target (Bruker) and was air-dried. The sample was then analyzed on the mass spectrometer with a laser intensity and detector gain kept constant at 60% laser and 3x, respectively. The raw spectra were exported as an '.ascii' file and plotted using Origin 8.5 software.

Western blot of MsaB

To measure the expression level of MsaB across the growth phases, quantitative western blot was performed with whole cell lysates. Overnight cultures were normalized to OD600= 0.05 and incubated at 37°C. Cells were harvested at OD600 = 0.7 (early), 1.5 (mid), 4.0 (late) and overnight (Post) points and frozen until used. Pellets were resuspended in PBS with protease inhibitor and lysed by bead beating. Crude lysates were centrifuged to remove the cell debris. Clear supernatants were collected and protein concentrations in them were determined by BCA method using PierceTM BCA protein assay kit (Life technologies, Grand Island, USA). 25 μg of proteins from each sample was loaded in SDS-Polyacrylamide gel and separated. After proteins were blotted to a PVDF membrane, and blocked by 5% non-fat skim milk, MsaB was detected by anti-MsaB antibody and peroxidase conjugated secondary antibody. MsaB bands were quantified in ImageJ software (67).

Stress Survival Assay

Survival capability of *S. aureus* during stress conditions was measured by enumerating the colony-forming units (CFUs) in different stress conditions. Briefly, overnight cultures were normalized to OD600= 0.05 and incubated at 37°C in TSB for 2 hours. Cells were collected by centrifugation and the spent media was discarded. Cells were then resuspended in either plain TSB as a control, or stress conditions such as, low pH (5.5), Saline condition (100 mM NaCl), and oxidative stress (10 mM H_2O_2). Cells were then incubated at 37°C. A sample of the cultures were collected every two hours, serially diluted, and spread on plain TSA plates. Next day, colonies were counted and plotted in logarithmic scale using GraphPad Prism software.

RNA extraction, reverse transcription, and qRT-PCR

An aliquot of an overnight culture was normalized to OD_{600} = 0.05 and grown to exponential phase. At this time, cells were subjected to either plain TSB or stress conditions such as, low pH (pH 5.0); saline stress; or 10 mM H_2O_2. Cells were grown for 1 h and harvested by centrifugation. The bacterial pellet was treated with RNAprotect™ Bacteria Reagent (Qiagen, Valencia, CA, USA) and stored at −80°C until used. RNA from the cell pellet was isolated using the RNeasy® mini kit (Qiagen) and dissolved in DEPC-treated H_2O. RNA quality was analyzed by determining the $A_{260/280}$ ratio using a nanodrop spectrophotometer (Thermo). Reverse transcription was carried out from 1000 ng of RNA using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Real-time PCRs were performed in triplicate using the primers listed in table 5.1. The fold change in gene expression was calculated using the formula $2^{-\Delta\Delta ct}$ (68), using the *gyrA* gene as an internal control. Fold change values were statistically analyzed by the independent sample t test using GraphPad prism software.

Results

MsaB exists as a dimer in solution

Recombinant His_6Halo-MsaB protein was purified by Nickel column using 250 mM imidazole as eluting agent. Upon analysis by PAGE, this protein appeared as a 42 kDa protein. Upon cleavage by TEV protease, a bigger fragment of 35 kDa and a smaller fragment of 15 kDa appeared in gel (Figure 5.2 A). Albeit in a very low quantity, the original 42 kDa protein also was found in the gel. The 35 kDa protein was predicted to be the His_6Halo tag based on bioinformatic analysis of its expected molecular weight (MW).

The smaller band was expected to be MsaB, however its molecular weight appeared to be exactly twice as much (15 kDa) as the predicted molecular weight of MsaB (7.5 kDa). This band was excised and analyzed by mass-spectrometry using a commercial source. Indeed, this protein was identified to be MsaB. To my surprise, MsaB appeared in gel as a dimeric protein, and was found to be resistant to boiling and detergent treatment. Therefore I hypothesized that MsaB exists as a dimer in solution. In HPLC chromatogram, fractions 7–8; 11; and 14–15 showed sharp peaks at 280 nm (Figure 5.2 B). These fractions were collected and analyzed in PAGE. Among these, Fraction 11 was found to be the 15 kDa protein, hence MsaB (Figure 5.2 C, D). After removal of acetonitrile removal, MsaB was dissolved in 25 mM Tris-HCl buffer, pH 7.4. As mentioned before, *msaB* ORF was cloned into the expression vector pH6HTNHis$_6$HaloTag® T$_7$ plasmid, which has a TEV protease cleavage site between the tag sequence and the *msaB* sequence. However, even after cleavage, MsaB carries over a small remnant of the vector sequence, which makes the molecular weight of recombinant MsaB precisely to be 9.2 kDa. Indeed, MALDI-ToF spectrum for MsaB revealed a peak at 9218.69 Da, which corresponds to the monomeric MsaB. However MALDI-ToF also revealed multiple peaks at 18447.85, 27643.53, and 36771.02 Da correspond to dimeric, trimeric, and tetrameric MsaB protein, respectively (Figure 5.2 E). The decreasing intensities of the multimeric peaks is likely due to the diminished ability of the higher order multimers to fly from the matrix. These peaks corresponding to multimers may also indicate that they are non-covalent oligomers of MsaB as indicated by the SDS-PAGE analysis. However, since dimer was found to be the only visible species in polyacrylamide gel, it suggests that MsaB predominantly exists in dimeric form in

solution, however monomeric, trimeric, and tetrameric species can also exist although in a very low quantities.

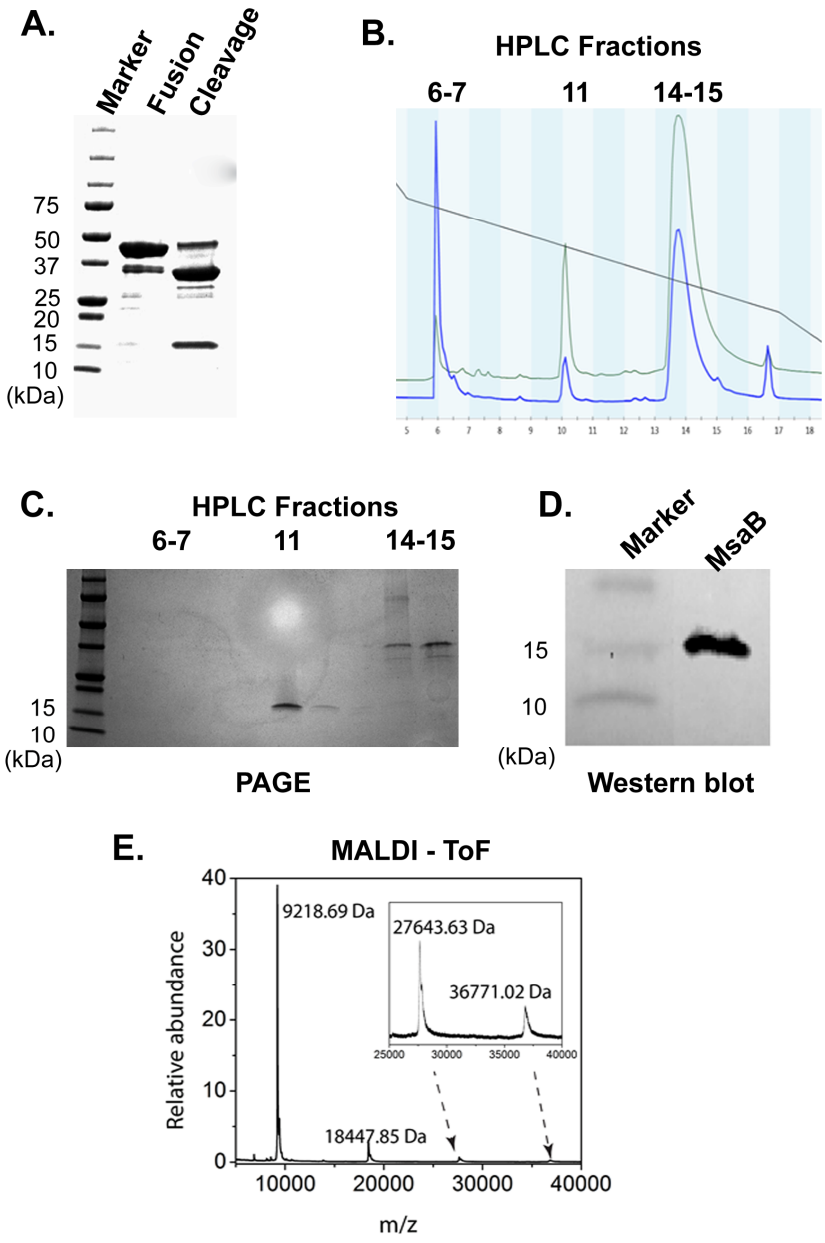

Figure 5.2. Expression, purification, cleavage, HPLC purification and MALDI-ToF analysis of MsaB protein: PAGE (A & C), Western blot (D), and MALDI-ToF (E) reveals that MsaB exists as predominantly dimeric species in solution.

MsaB consists of predominantly α – helical conformation in solution

The secondary structure of recombinant MsaB was analyzed using far-UV circular dichroism (CD). The spectrum showed that the protein adopted a α-helical conformation with a signature negative minimum at 208 nm and a shoulder at 222 nm (Figure 5.3). Given the expected transcriptional regulator activity of MsaB, it is not surprising to observe this conformation, as most DNA binding protein regulators form α-helix, as they bind and regulate gene expression via the classical helix-turn-helix motif (129, 130).

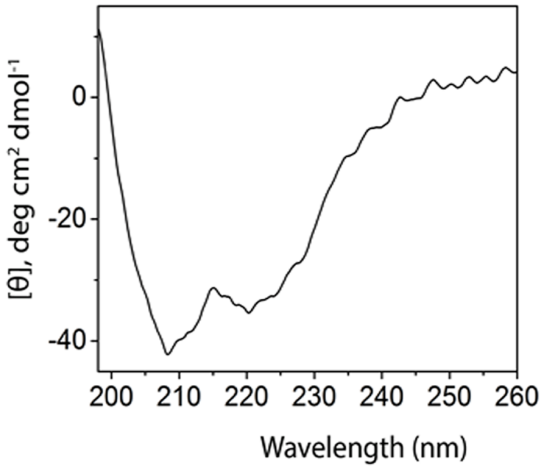

Figure 5.3. Circular Dichroism spectrum reveals that MsaB adopts an alpha-helical structure in solution.

MsaB exists as a dimer in vivo and its expression is temporally regulated

Our results of analysis of MsaB in solution suggest that MsaB perhaps exists as a dimeric species. However, whether this was an effect of a reagent or any artificial condition on MsaB's structure, was not clear. To eliminate this possibility, I examined the molecular weight of MsaB from whole cell lysates of two *S. aureus* strains – Mu50 and UAMS-1 by western blot using anti-MsaB antibody throughout the different phases of growth. Western blot of the MsaB protein in the early, mid, late, and post–exponential

phases of growth revealed that MsaB indeed exists as a dimer in vivo as well (Figure 4.4). Western blot also suggests that although MsaB is expressed in all phases of growth in both Mu50 and UAMS-1, its expression level decreases over time and is expressed less in the late and post phases of growth compared to the early phases.

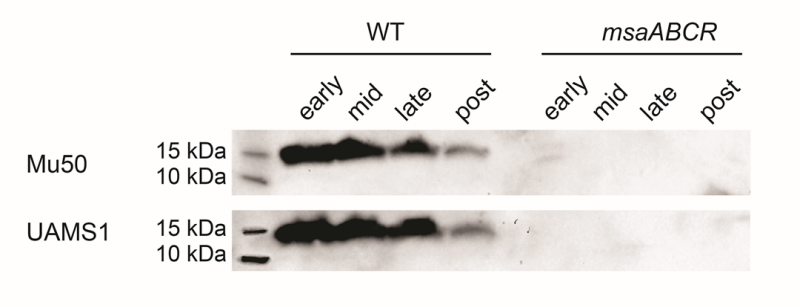

Figure 5.4. Western blot of MsaB at different growth phases in Mu50 and UAMS-1

Expression of msaB is up-regulated during stress conditions:

As hypothesized before, that MsaB, being a CspA family protein may be involved in stress regulation in *S. aureus.* Real time PCR with RNA isolated from cells subjected to stress conditions revealed that the expression of *msaB* gene is up regulated 2.5 fold in oxidative stress (H_2O_2), 4.5 fold in vancomycin stress, and 3 fold in low pH. However in saline stress, no change in expression of *msaB* was noticed. These results suggest that MsaB may indeed be involved in stress response regulation in *S. aureus.*

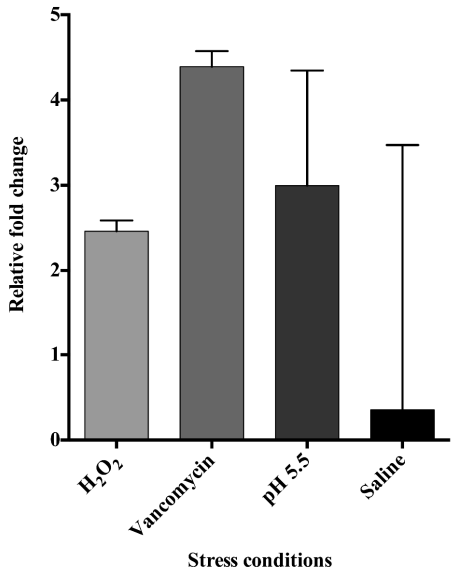

Figure 5.5. RT-qPCR revealed that *msaB* is upregulated during stress condition.

msaABCR mutant has altered expression of RNA – polymearase subunits

I previously reported that Sigma B (σ^B), which is an important component of *S. aureus* RNA polymersase, is down-regulated in the *msaABCR* mutant of the VISA strain Mu50 (131).

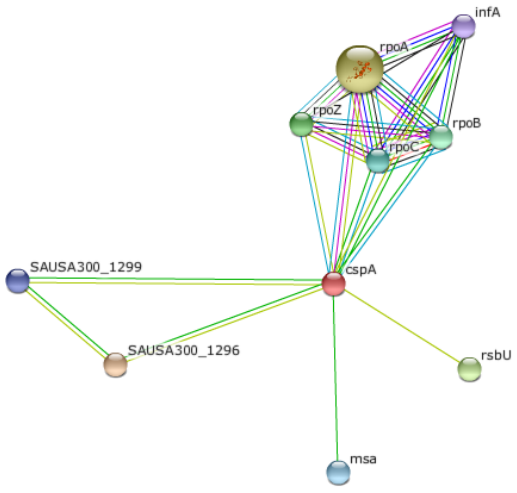

Figure 5.6. STRING analyses revealed that MsaB is closely linked to RNA polymerase subunits.

Similar observation was also done by others (56) in the strain USA300 LAC. RNA polymerases have been known to be responsible for altering global expression of genes during stress conditions (23, 132). It has been reported that cold shock family protein CspC regulates an RNA polymerase subunit RpoS in *E. coli* to regulate stress response (23). Moreover from bioinformatic prediction analysis performed in the web based tool STRING (http://string-db.org/), MsaB was found to be closely linked to RpoA, RpoB, RpoC, RpoZ, and SigmaB (Figure 5.6). Hence I tested the transcription level of *rpoA, rpoB, rpoC,* and *rpoZ* in wild type and *msaABCR* mutants, in both plain and stress conditions. qRT-PCR analysis revealed that wild type and *msaABCR* mutant has drastically different expression pattern of *rpo* genes. For instance, *rpoZ* is severely downregulated in saline stress in the mutant, whereas its expression is unchanged in the wild type. Also, *rpoA* is drastically downregulated in low pH in the wild type, but relatively unchanged in the mutant. Moreover *rpoB, rpoC,* and *rpoZ* are all upregulated in both oxidative stress and vancomycin stress, however they are relatively unresponsive to these conditions in the mutant (Figure 5.7).

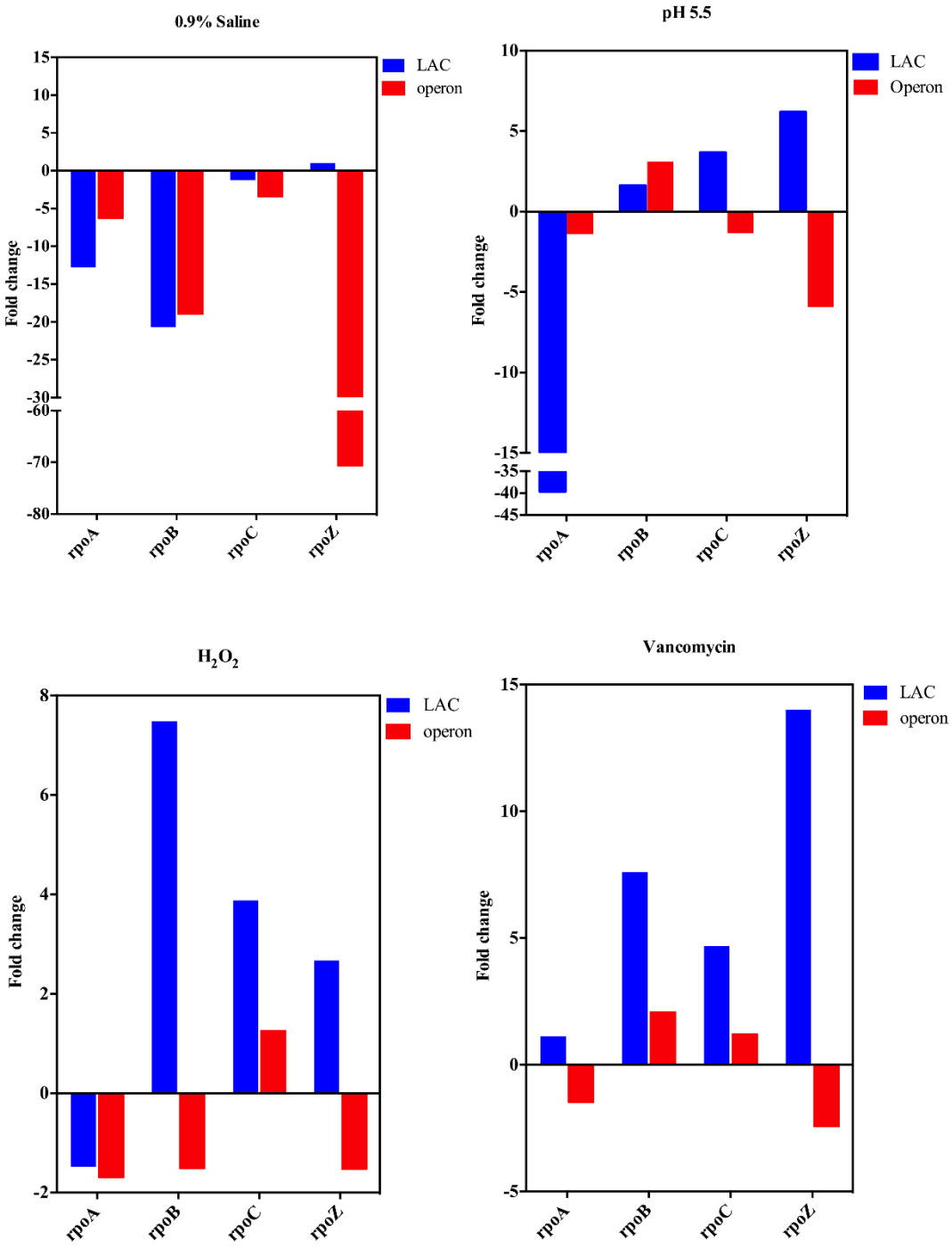

Figure 5.7. qRT-PCR of *rpo* genes revealed a differential expression pattern between the wild type and the *msaABCR* mutant during different stress conditions

Discussion

MsaB, being a cold shock family protein, was hypothesized to be a stress response regulator protein in *S. aureus*. In this part of my study, I investigated the molecular structure of MsaB protein and its possible role of MsaB in stress response regulation. To my knowledge this is the first study to systematically characterize the structure and function of MsaB protein in *S. aureus*. Biophysical analysis of MsaB and polyacrylamide gel electrophoresis revealed that it exists primarily as dimeric species in solution. The reason for the multimeric nature of the MsaB protein is not clear at the moment. Most DNA binding proteins have been found to dimerize at the binding site, however they usually do not produce dimer in solution (133). MsaB was found to produce a strong dimer which is resistant to SDS denaturation. The reason behind this is also unclear and experiments are underway to investigate this phenomenon. Another interesting fact that was revealed in the biophysical analysis is that MsaB has a predominantly α-helical structure in solution. This is contrasting to most of the cold shock family proteins that are composed of mostly anti-parallel β-sheets. Three – dimensional structure of *E. coli* CspA, *Bacillus subtilis–, B. caldolyticus–,* and *Thermotoga maritime–* CspB all revealed presence of five anti-parallel β-strands that form a β-barrel (134). Moreover many other proteins that carry cold shock domains possess the same β-barrel structure. The eukaryotic Y-box proteins, that also contain this barrel structure has also been shown to be DNA/RNA binding protein. Many of these cold shock proteins bind to single stranded DNA (ssDNA) or RNA (ssRNA). The only exception is eukaryotic Clah8, which can bind to double-stranded DNA (dsDNA). In a parallel study in our laboratory my colleague discovered that MsaB is able to bind

dsDNA and consequently regulates the transcription of the target gene. This data suggests that MsaB also has dsDNA binding properties like eukaryotic Clah8 protein. Our observation of MsaB possessing predominantly α-helix suggests that MsaB may be a member of helix-turn-helix DNA binding protein family rather than conventional β-barrel possessing cold shock proteins. Later in this study, MsaB protein was also found to be involved in stress response in *S. aureus*. *msaB* gene was found to be upregulated in all tested stress conditions except saline. This suggests *msaB* may be an essential gene to regulate transcription of other genes during the stress. Cold shock proteins are known to regulate sigma factors. In the chapter II, in my study with vancomycin resistance, it was found that sigma B gene was significantly down-regulated in the *msaABCR* mutant of VISA strain Mu50. Also, our observation of alteration in expression of *rpo* genes suggest that MsaB might be regulating the transcription of the RNA polymerase subunits including sigma factors to modulate global gene expression in *S. aureus*. Further investigation such as chromatin immune-precipitation (ChIP) coupled with high throughput sequencing (ChIP-Seq) using anti MsaB antibody, is currently being carried out to unravel which promoters MsaB binds to, in a global level to prepare the cells for survival during stress conditions such as the presence of antibiotic, peroxide, or low pH. Our earlier observations of defective biofilm, defective capsule, and defective antibiotic resistance in the *msaABCR* mutant may all be the result of absence of regulation of RNA polymerases by MsaB protein during these stress conditions. However more detailed investigations are required to pinpoint the role of MsaB protein in survival during the stress situations.

Table 5.1

Primers used in this study

Experiment	Primer name	Sequence (5' to 3')
qRT-PCR	*msaB* RT F	CGTATTCGTACATTTTTCAGCAA
	msaB RT R	CGGTCGCCTTCAACTACTTC
	rpoA RT F	AATTGGTGTAATCCCTGTTG
	rpoA RT R	ACCATTAGTCCAAACATCCA
	rpoB RT F	AAACAGCGAAGTGTTTGAAT
	rpoB RT R	GTACGACCTTCATCATCGTT
	rpoC RT F	CGCGACTGAAAGCTACTTAT
	rpoC RT R	GAACCATAACCTCAACGTGT
	rpoZ RT F	AAATTGATGAACAACCTGAAA
	rpoZ RT R	TACCGTCAGCAATTTCTTCT

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